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(54) **MEMBRANE-SEPARATION-TYPE CULTURE DEVICE, MEMBRANE-SEPARATION-TYPE CULTURE KIT, STEM CELL SEPARATION METHOD USING SAME, AND SEPARATION MEMBRANE**

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USPC .. **435/375**; 435/297.1; 435/297.5; 435/286.1; 435/308.1; 427/551

(57) **ABSTRACT**

A membrane separation culture device includes an upper structure including a vessel in which at least a portion of the bottom thereof is formed with a separation membrane having pores that allow stem cells to permeate therethrough, and a lower structure including a vessel that retains a fluid in which the membrane of the upper structure is immersed.

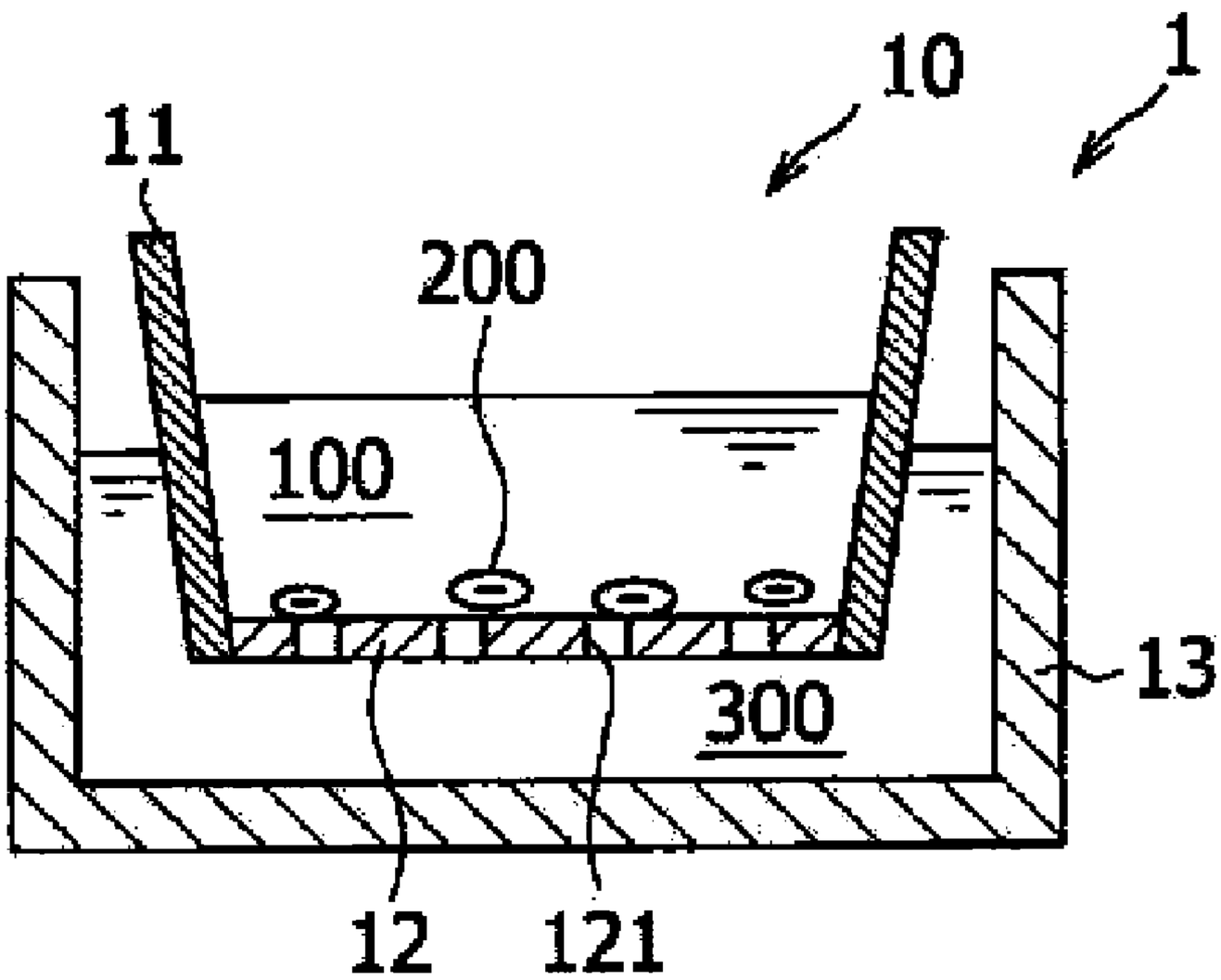


Fig. 1

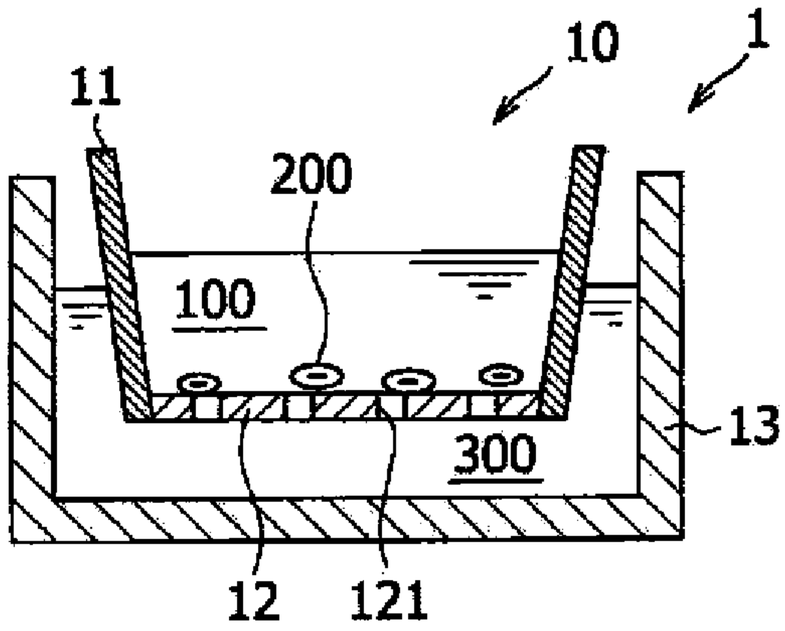

10
1
11
200
100
13
300
12
121

Fig. 2

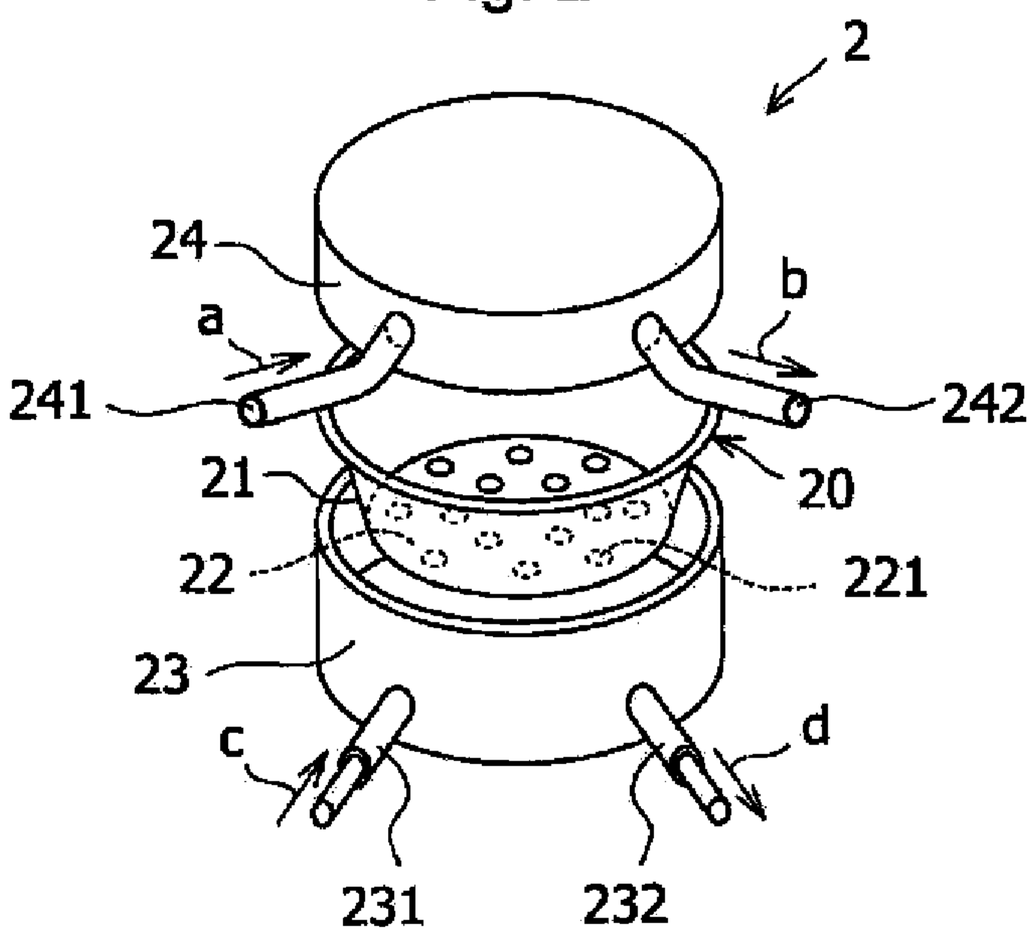

2
24
a
b
241
242
21
20
22
221
23
c
d
231
232

Fig. 3

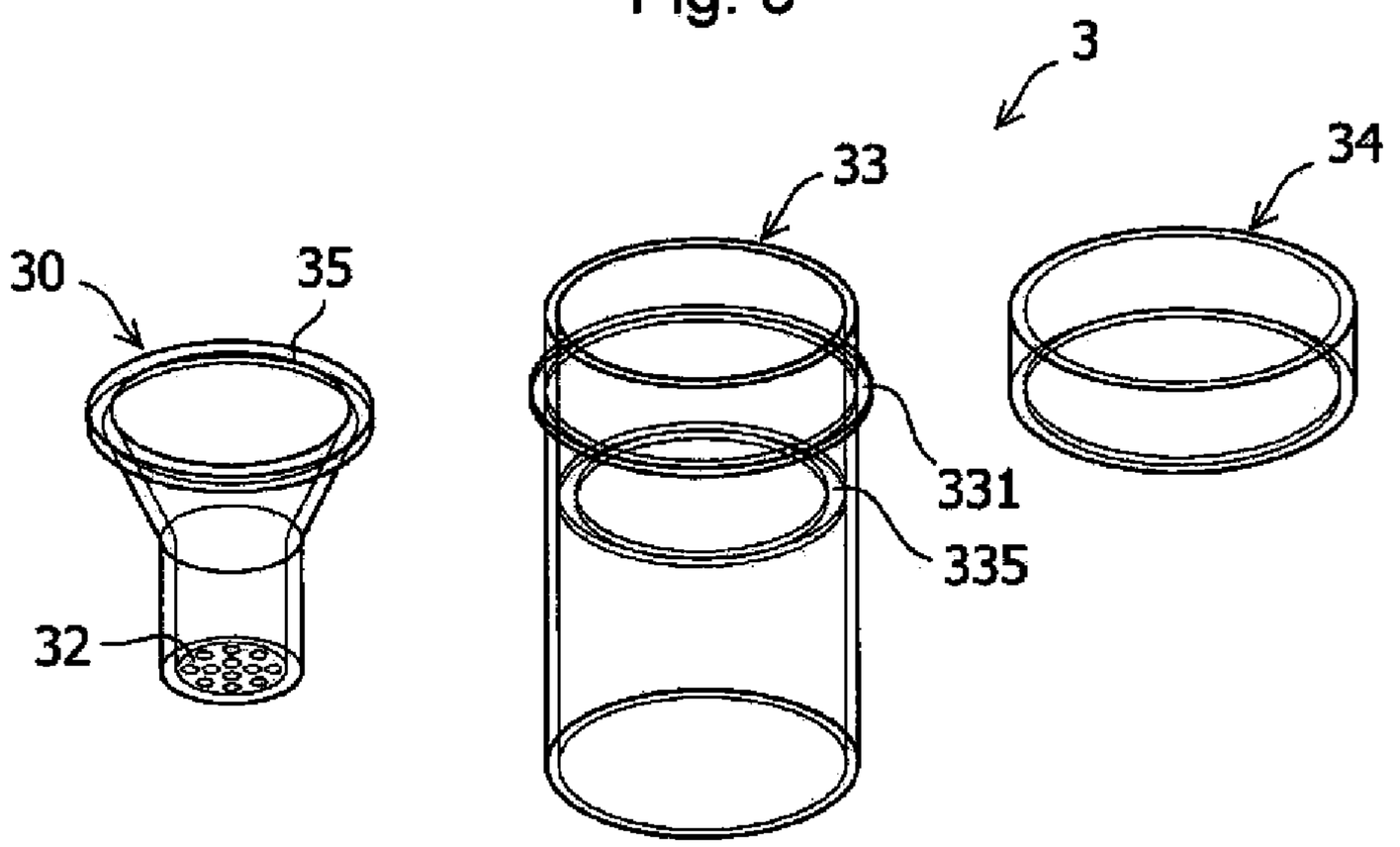


Fig. 4

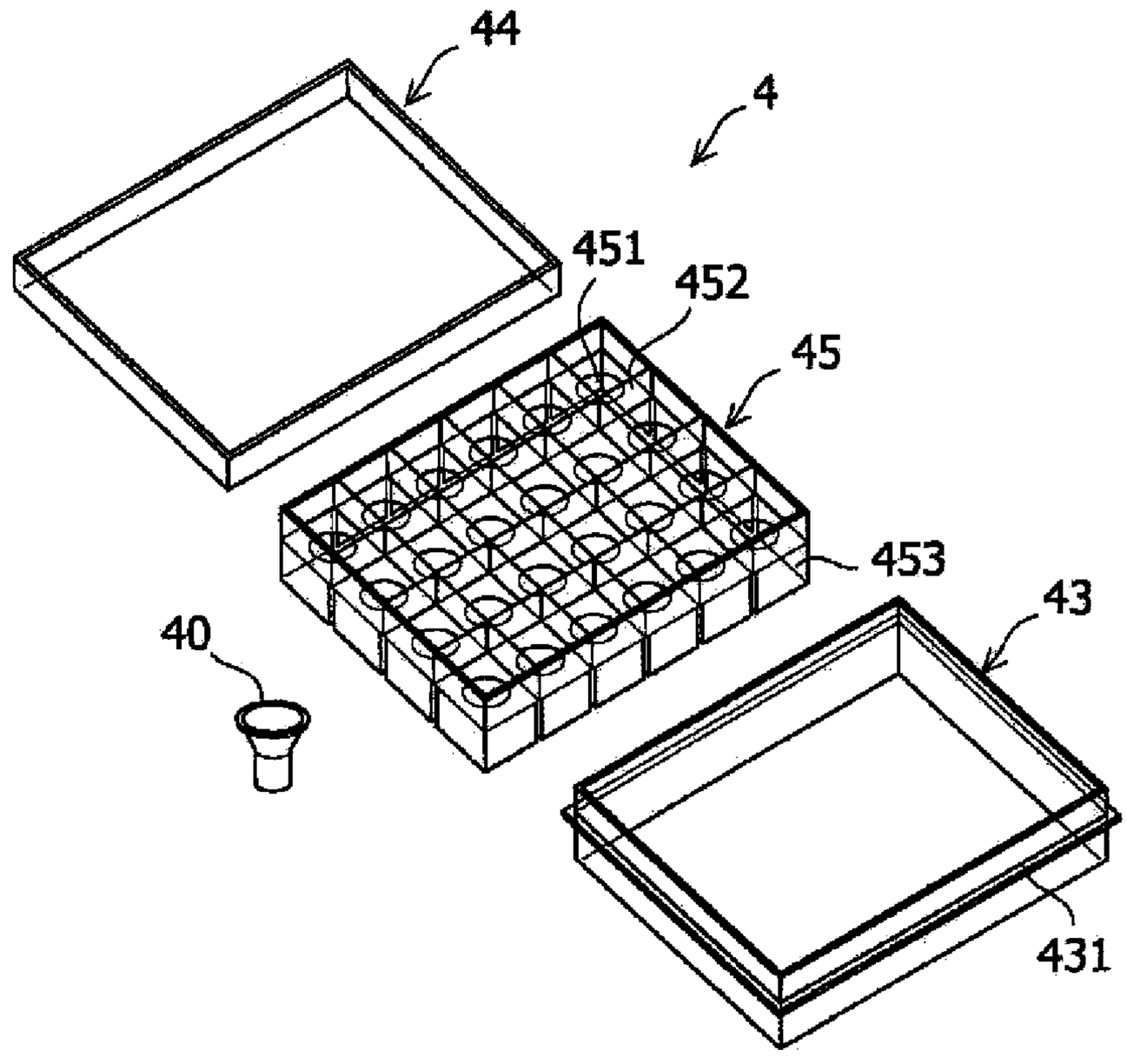


Fig. 5

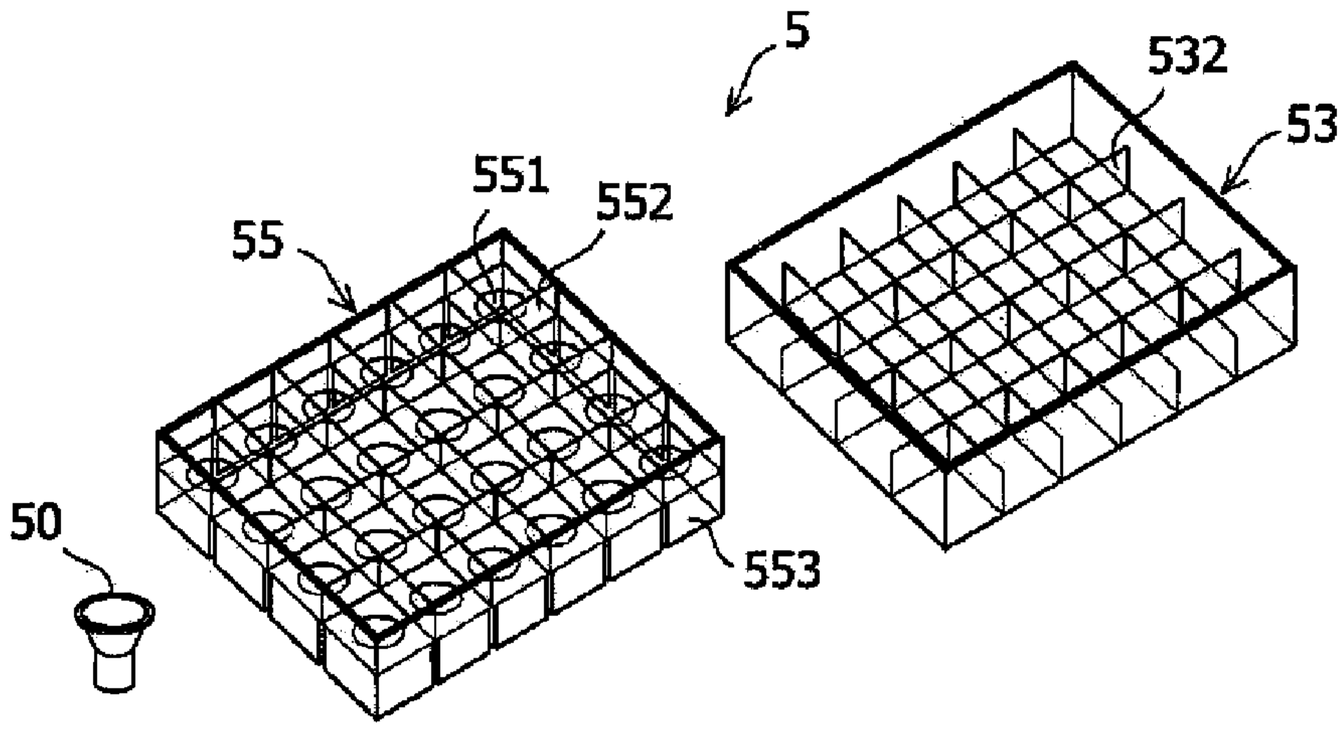
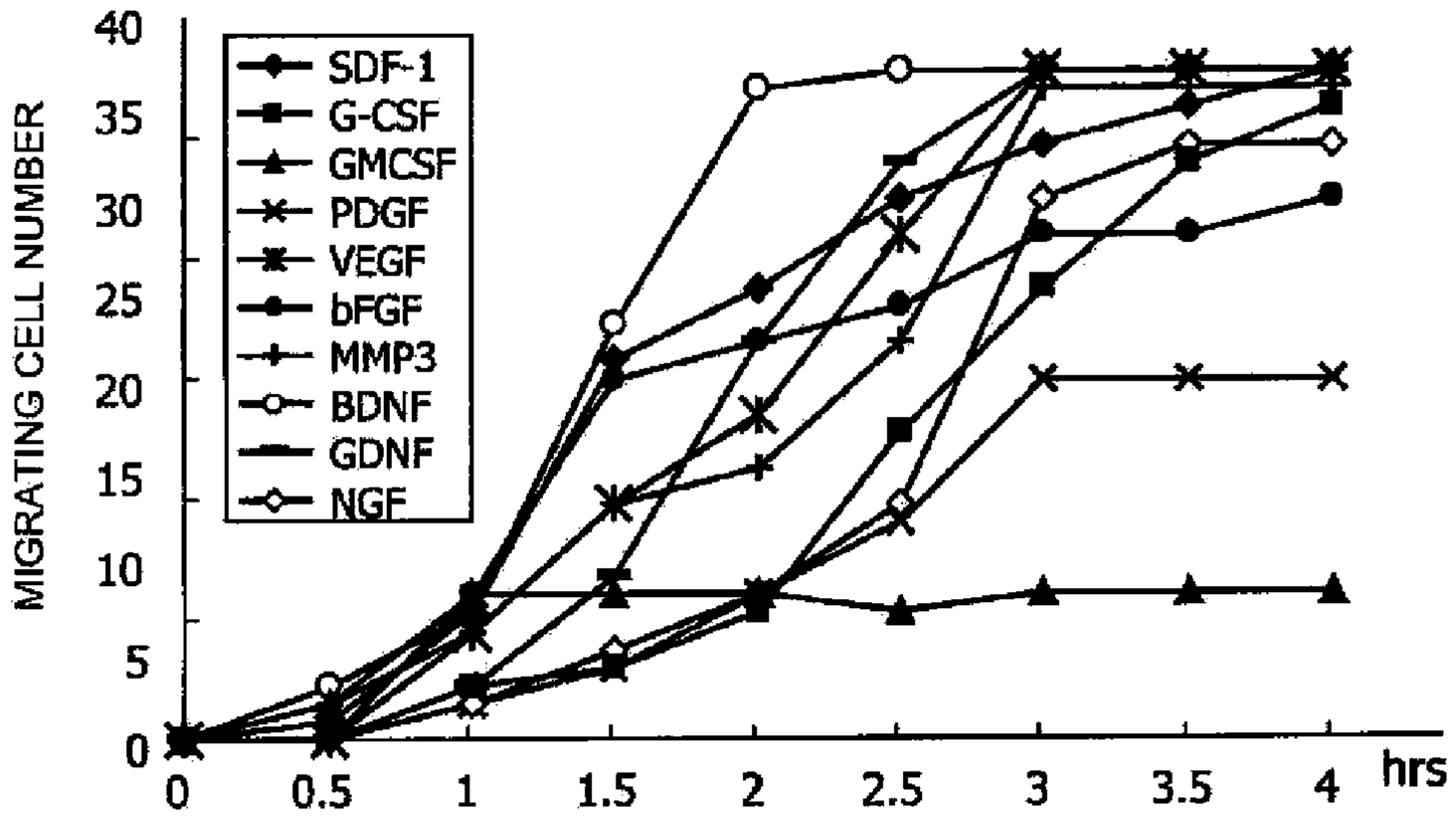


Fig. 6(a)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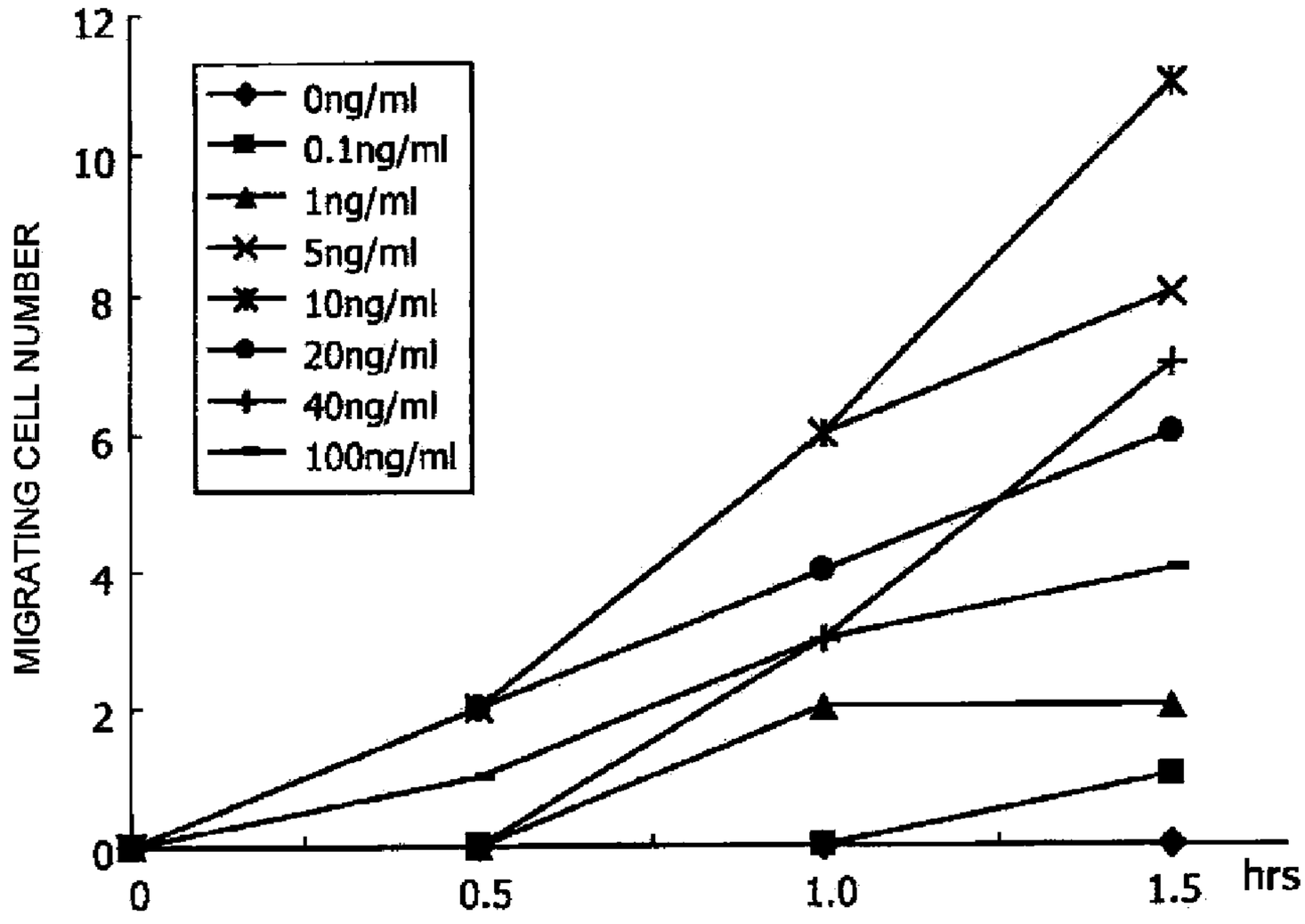
30
25
20
15
10
5
0
0
0.5
1
1.5
2
2.5
3
3.5
4
hrs
SDF-1
G-CSF
GMCSF
PDGF
VEGF
bFGF
MMP3
BDNF
GDNF
NGF

Fig. 6(b)


MIGRATING CELL NUMBER
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8
6
4
2
0
0
0.5
1.0
1.5
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0.1ng/ml
1ng/ml
5ng/ml
10ng/ml
20ng/ml
40ng/ml
100ng/ml

Fig. 7(a)

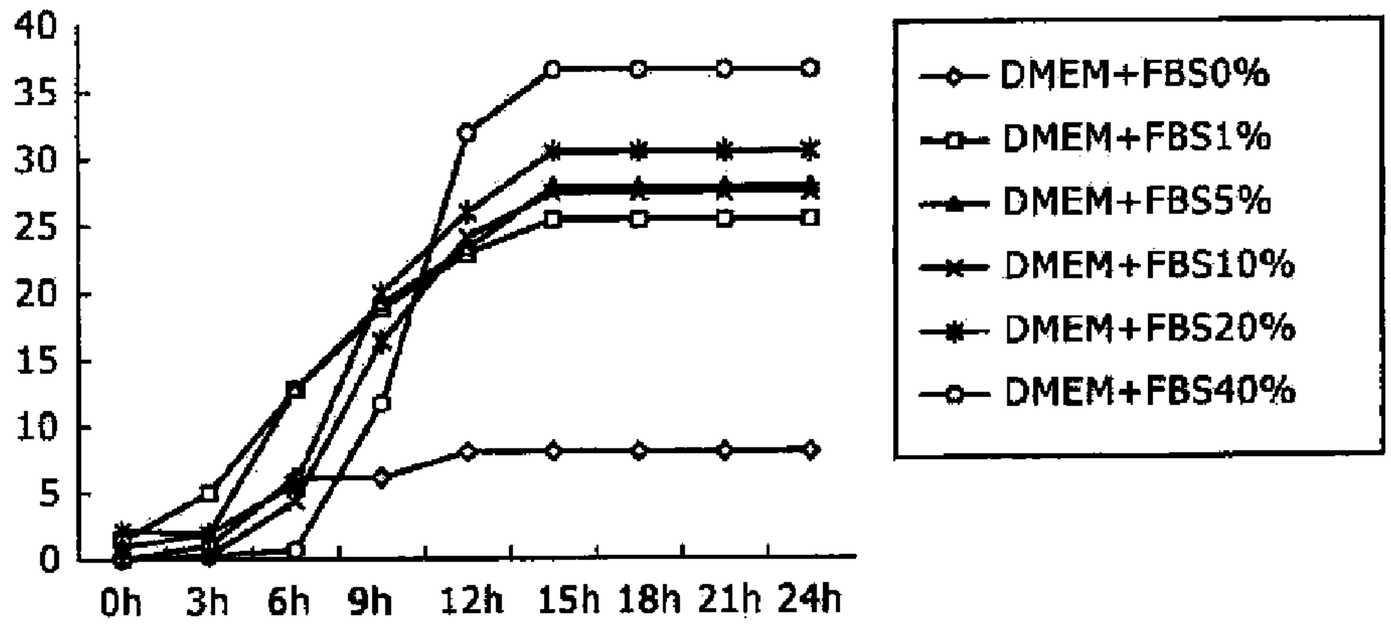
40
35
30
25
20
15
10
5
0
0h
3h
6h
9h
12h
15h
18h
21h
24h
DMEM+FBS0%
DMEM+FBS1%
DMEM+FBS5%
DMEM+FBS10%
DMEM+FBS20%
DMEM+FBS40%

Fig. 7(b)

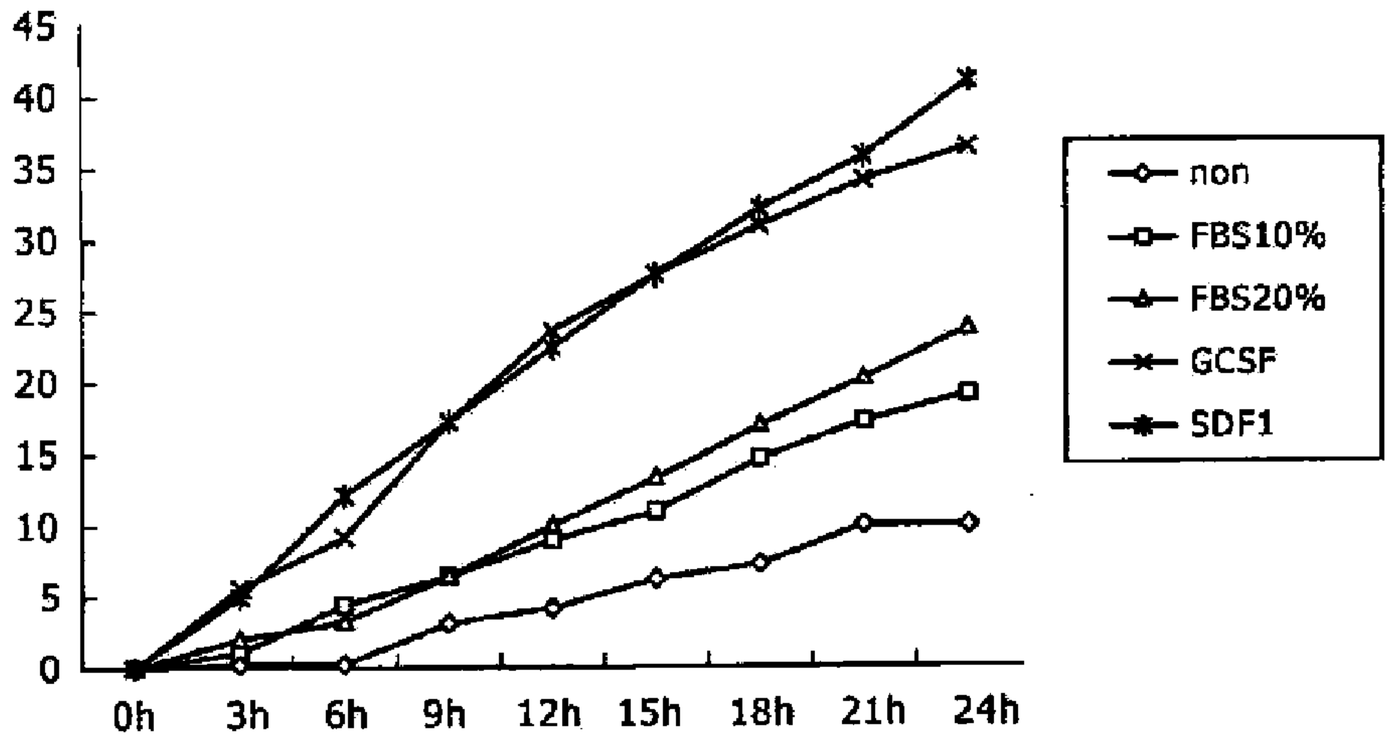
45
40
35
30
25
20
15
10
5
0
0h
3h
6h
9h
12h
15h
18h
21h
24h
non
FBS10%
FBS20%
GCSF
SDF1

Fig. 8(a)

Fig. 8(b)

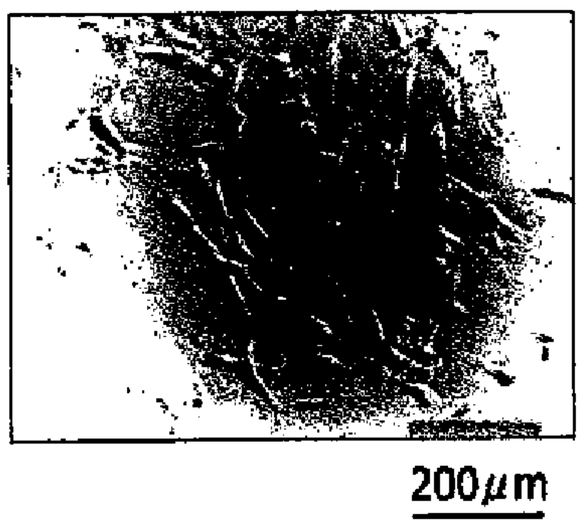

Fig. 8(c)

Fig. 9(a)

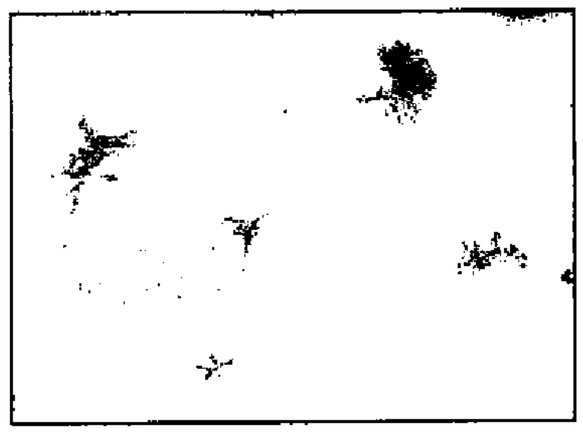

Fig. 9(b)

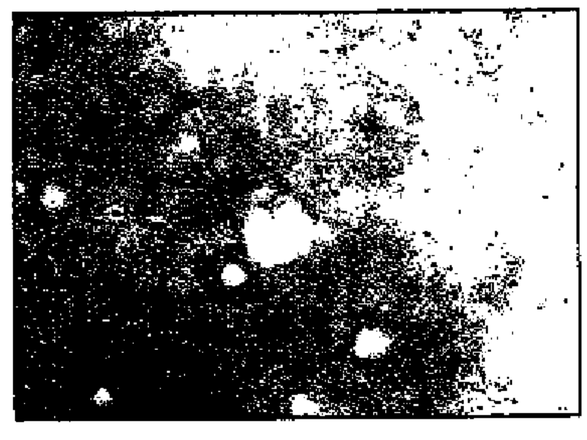

Fig. 10(a)

Fig. 10(b)

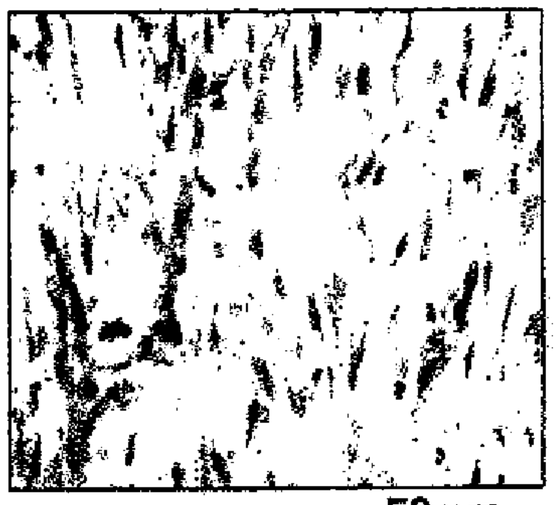

Fig. 10(c)

Fig. 10(d)

Fig. 11

Fig. 12(a)

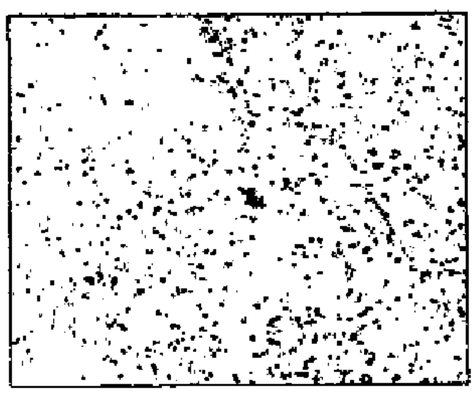

Fig. 12(b)

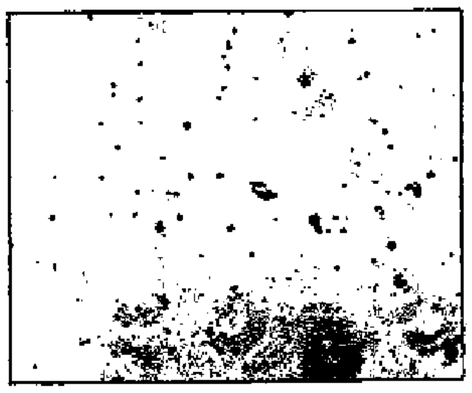

Fig. 12(c)

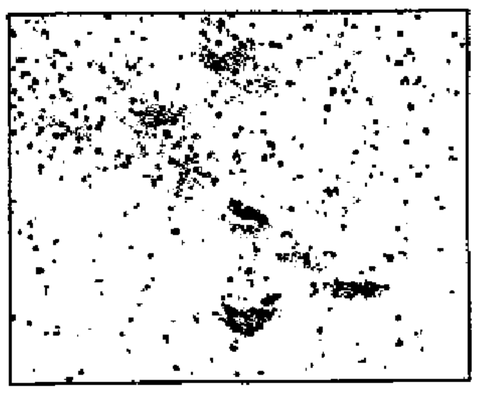

Fig. 12(d)

Fig. 12(e)

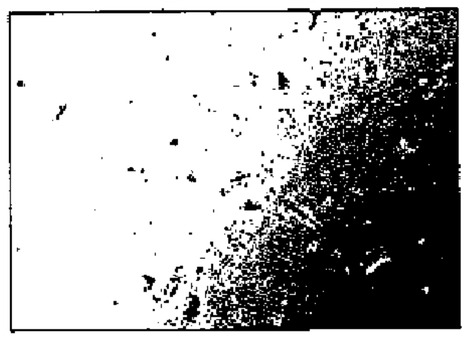

Fig. 13(a)

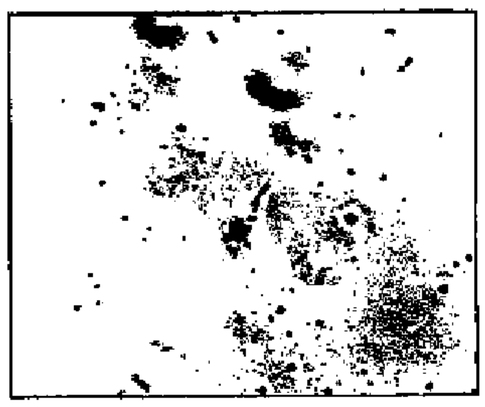

Fig. 13(b)

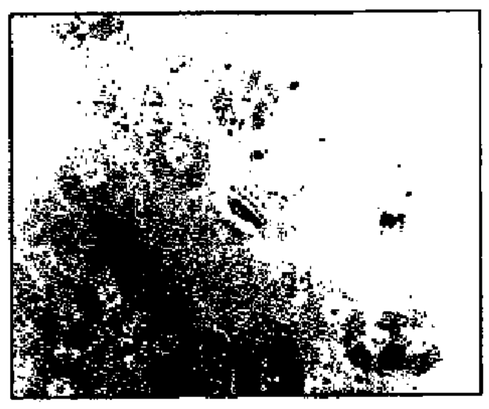

Fig. 13(c)

Fig. 13(d)

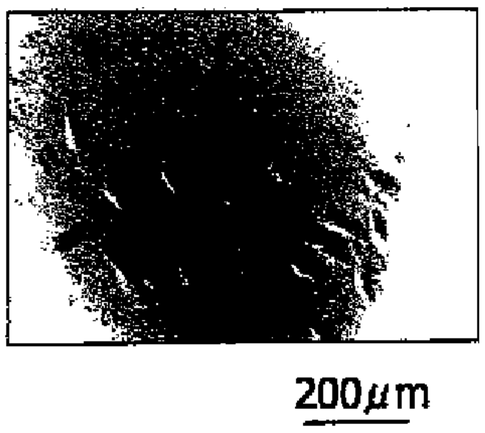

Fig. 14

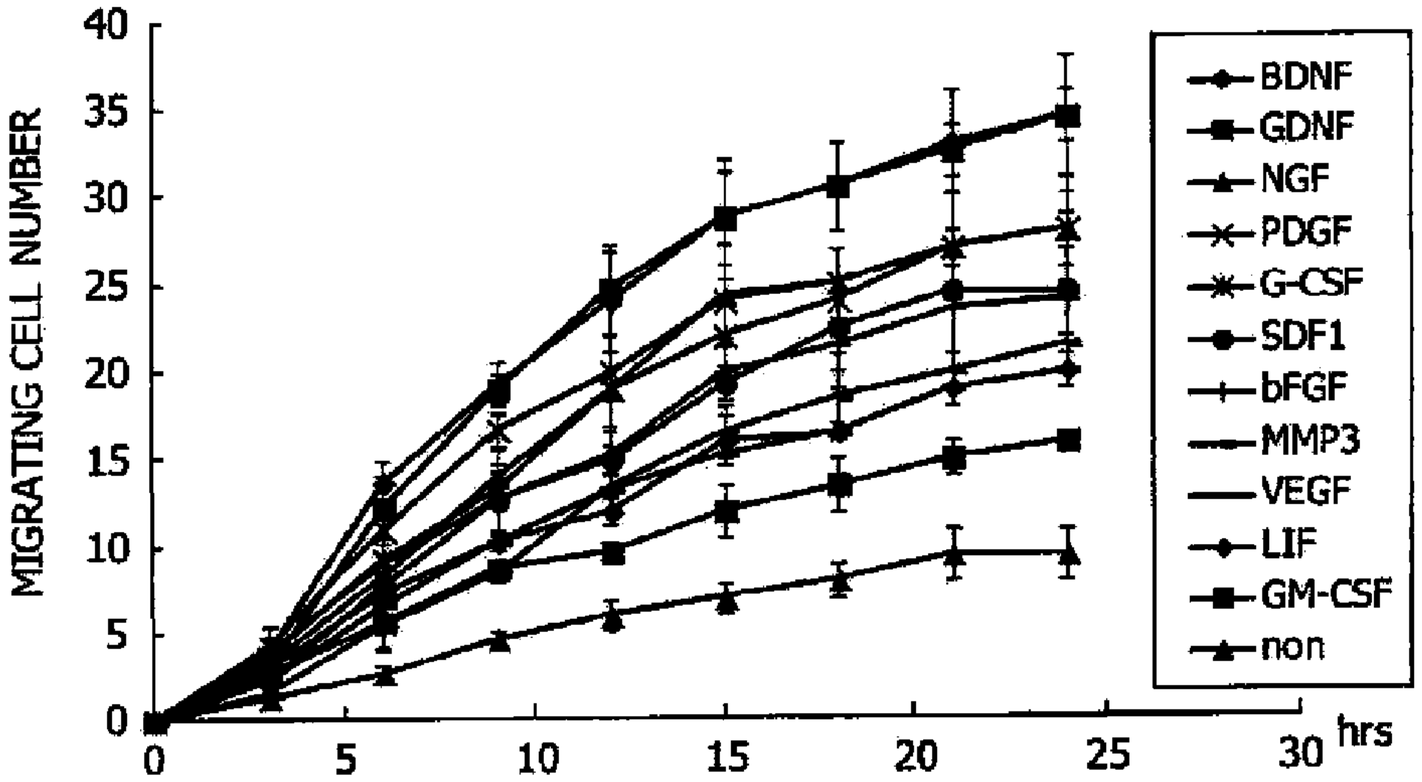

Fig. 15

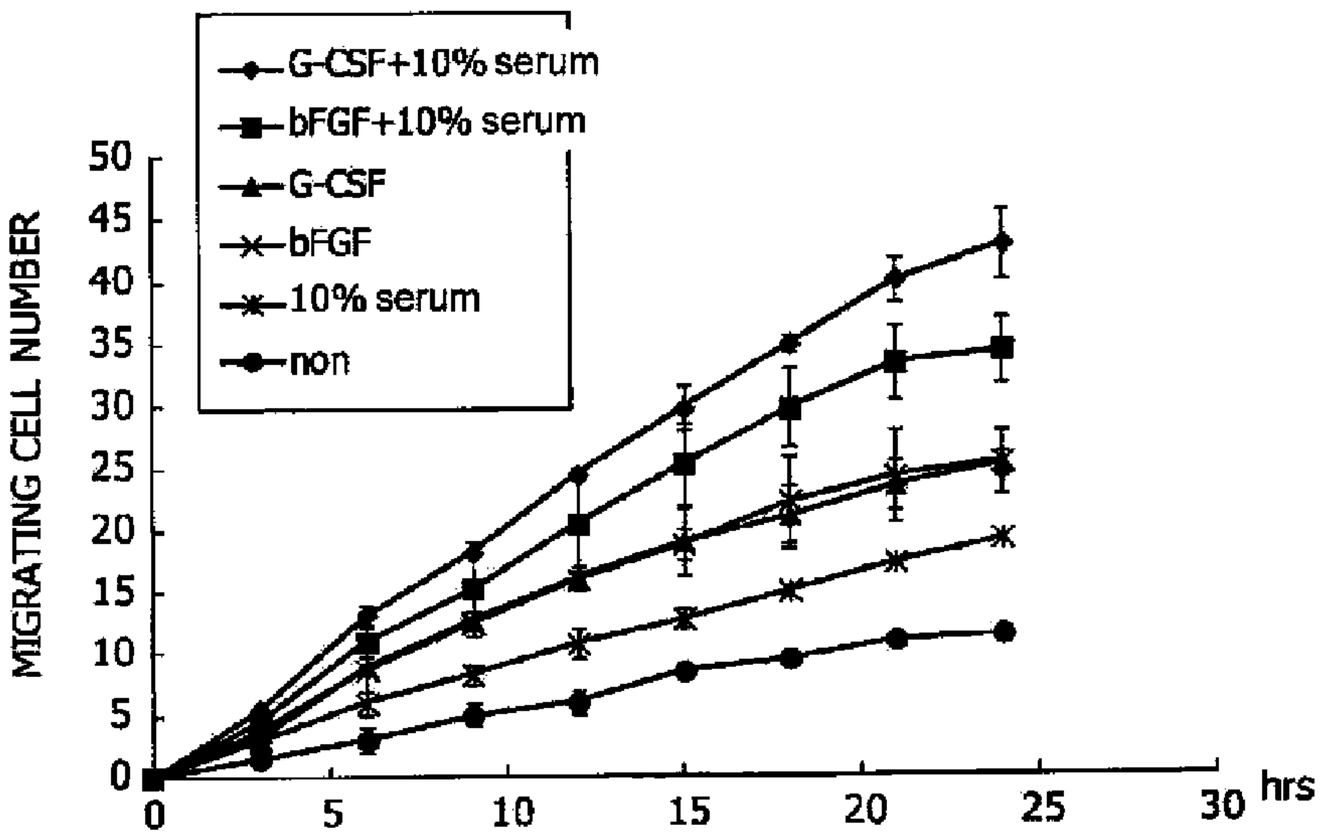

Fig. 16

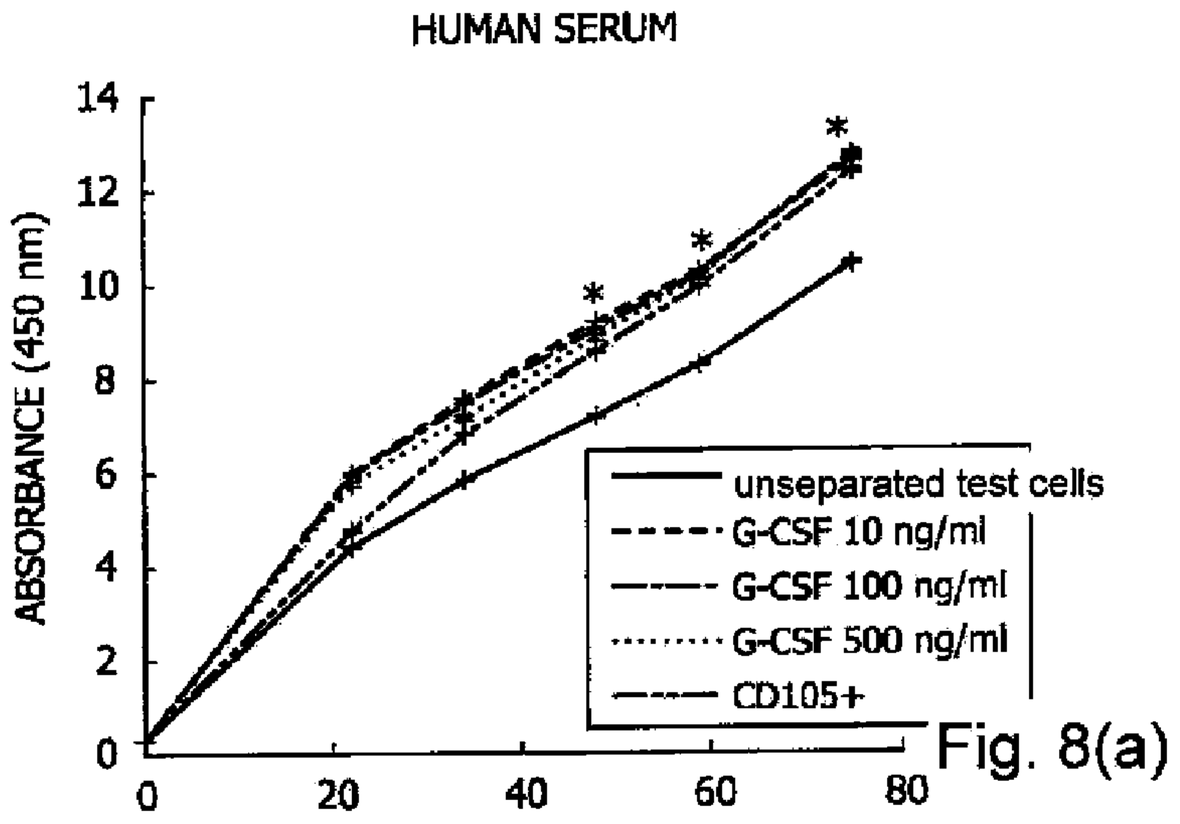

Fig. 8(a)

Fig. 17

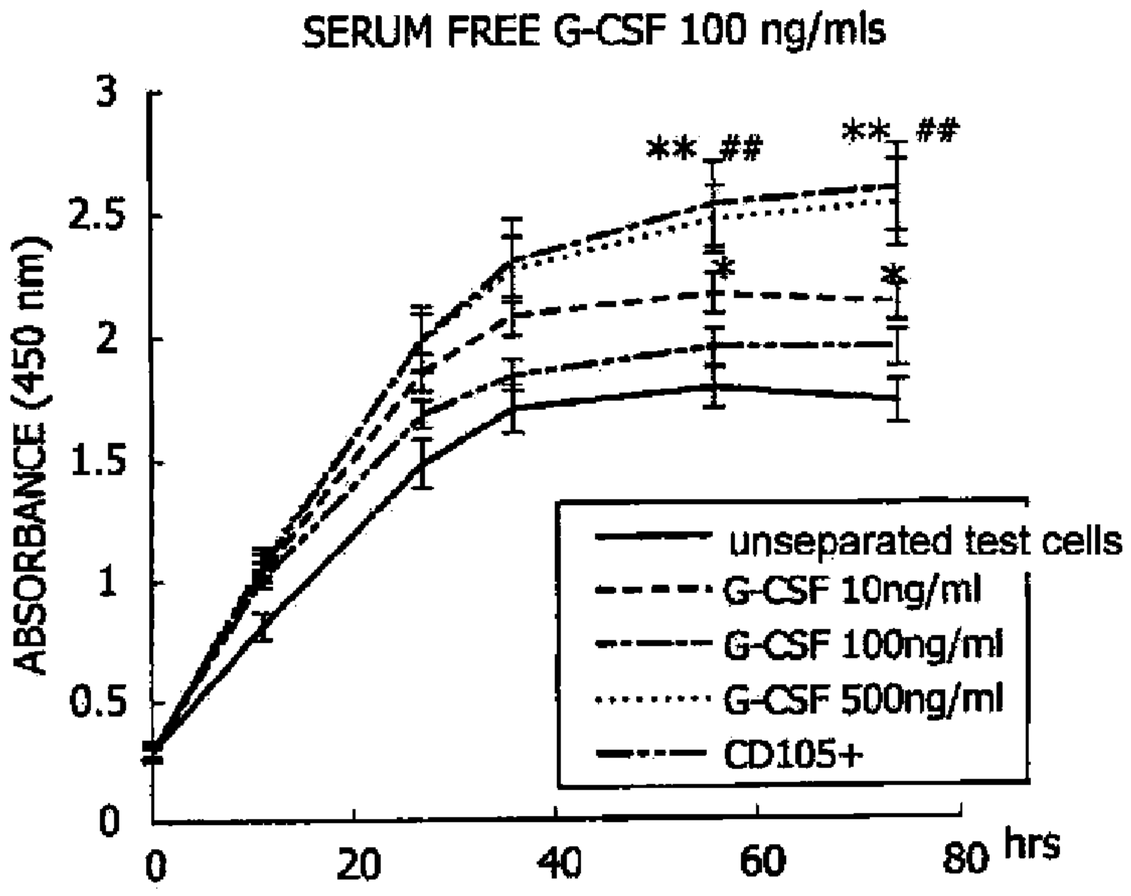
SERUM FREE G-CSF 100 ng/mls
ABSORBANCE (450 nm)
3
2.5
2
1.5
1
0.5
0
0
20
40
60
80
hrs
** ##
** ##
*
*
unseparated test cells
G-CSF 10ng/ml
G-CSF 100ng/ml
G-CSF 500ng/ml
CD105+

Fig. 18

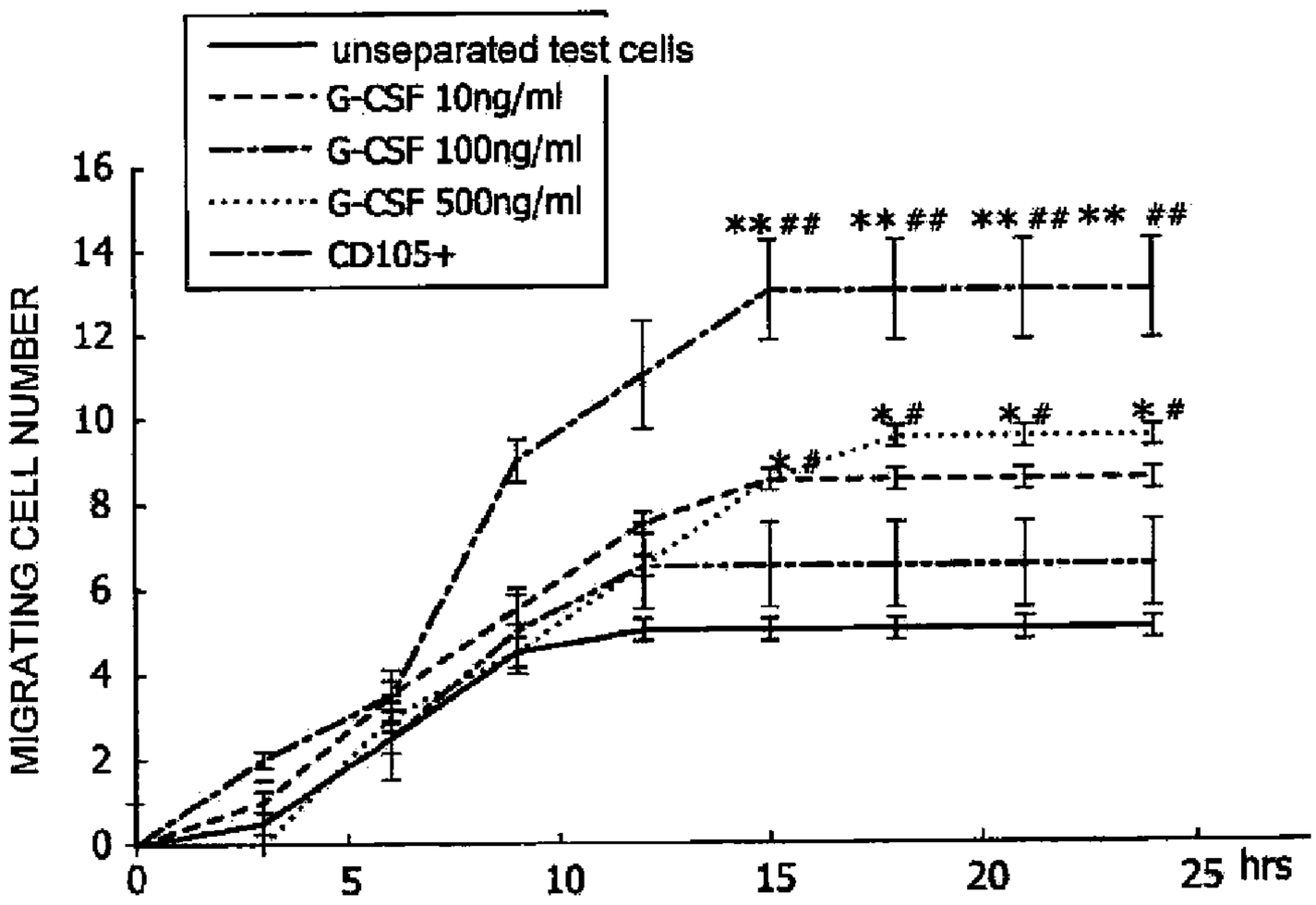
unseparated test cells
G-CSF 10ng/ml
G-CSF 100ng/ml
G-CSF 500ng/ml
CD105+
MIGRATING CELL NUMBER
16
14
12
10
8
6
4
2
0
0
5
10
15
20
25
hrs
** ## ** ## ** ## ** ##
*#
*#
*#
*#

Fig. 19

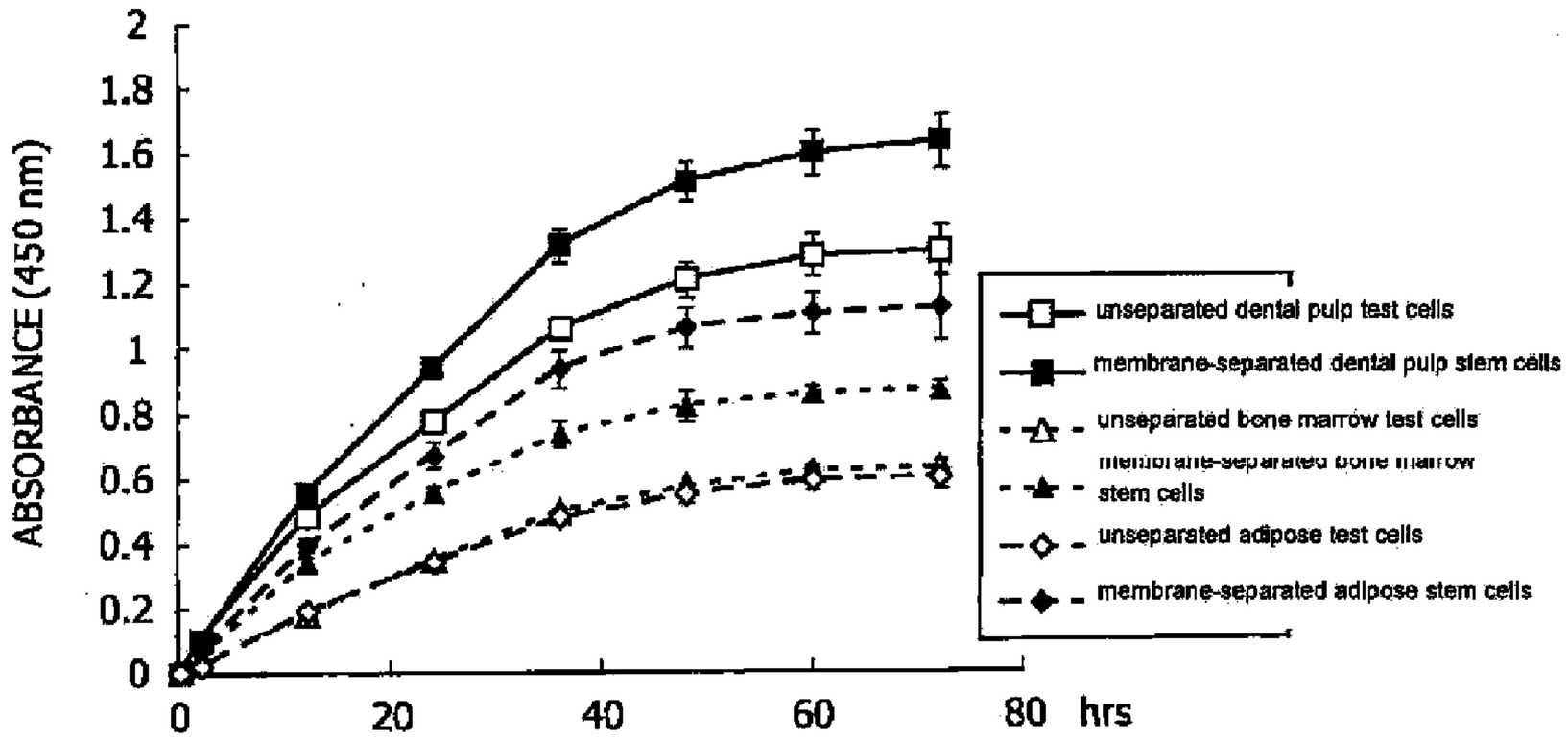
ABSORBANCE (450 nm)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
20
40
60
80
hrs
unseparated dental pulp test cells
membrane-separated dental pulp stem cells
unseparated bone marrow test cells
membrane-separated bone marrow stem cells
unseparated adipose test cells
membrane-separated adipose stem cells

Fig. 20

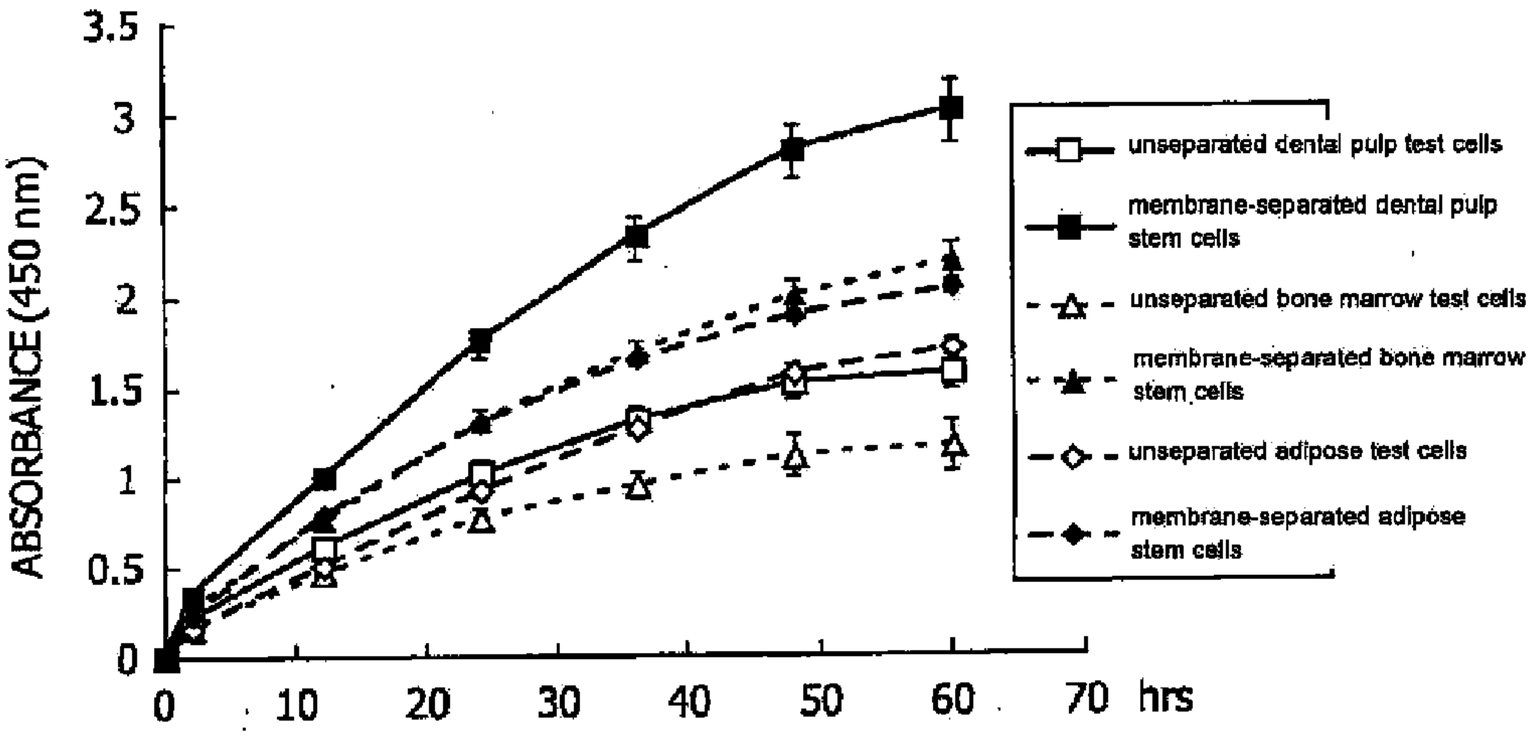
ABSORBANCE (450 nm)
3.5
3
2.5
2
1.5
1
0.5
0
0
10
20
30
40
50
60
70
hrs
unseparated dental pulp test cells
membrane-separated dental pulp stem cells
unseparated bone marrow test cells
membrane-separated bone marrow stem cells
unseparated adipose test cells
membrane-separated adipose stem cells

Fig. 21

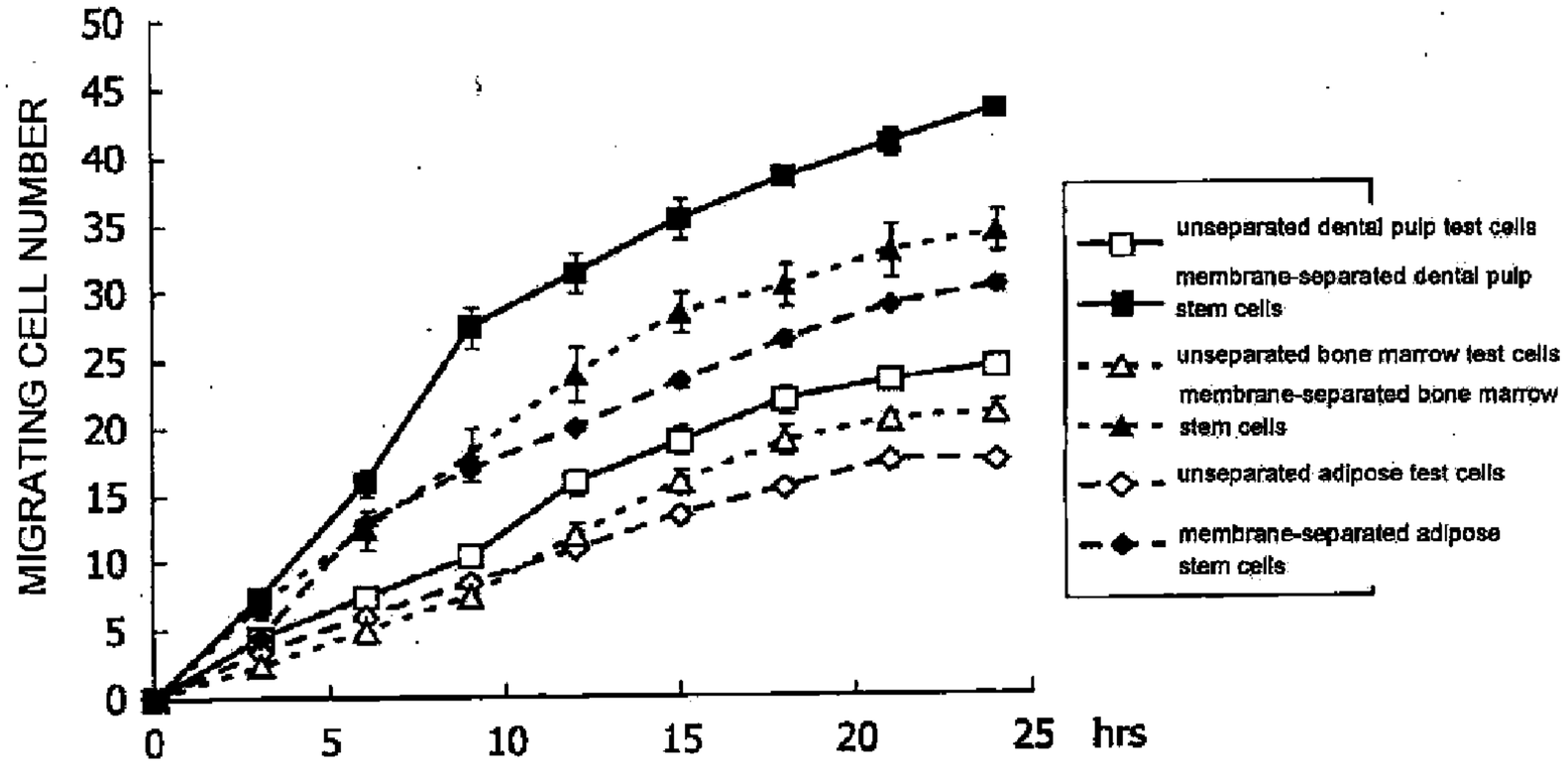
MIGRATING CELL NUMBER
50
45
40
35
30
25
20
15
10
5
0
0
5
10
15
20
25
hrs
unseparated dental pulp test cells
membrane-separated dental pulp stem cells
unseparated bone marrow test cells
membrane-separated bone marrow stem cells
unseparated adipose test cells
membrane-separated adipose stem cells

Fig. 22(a)

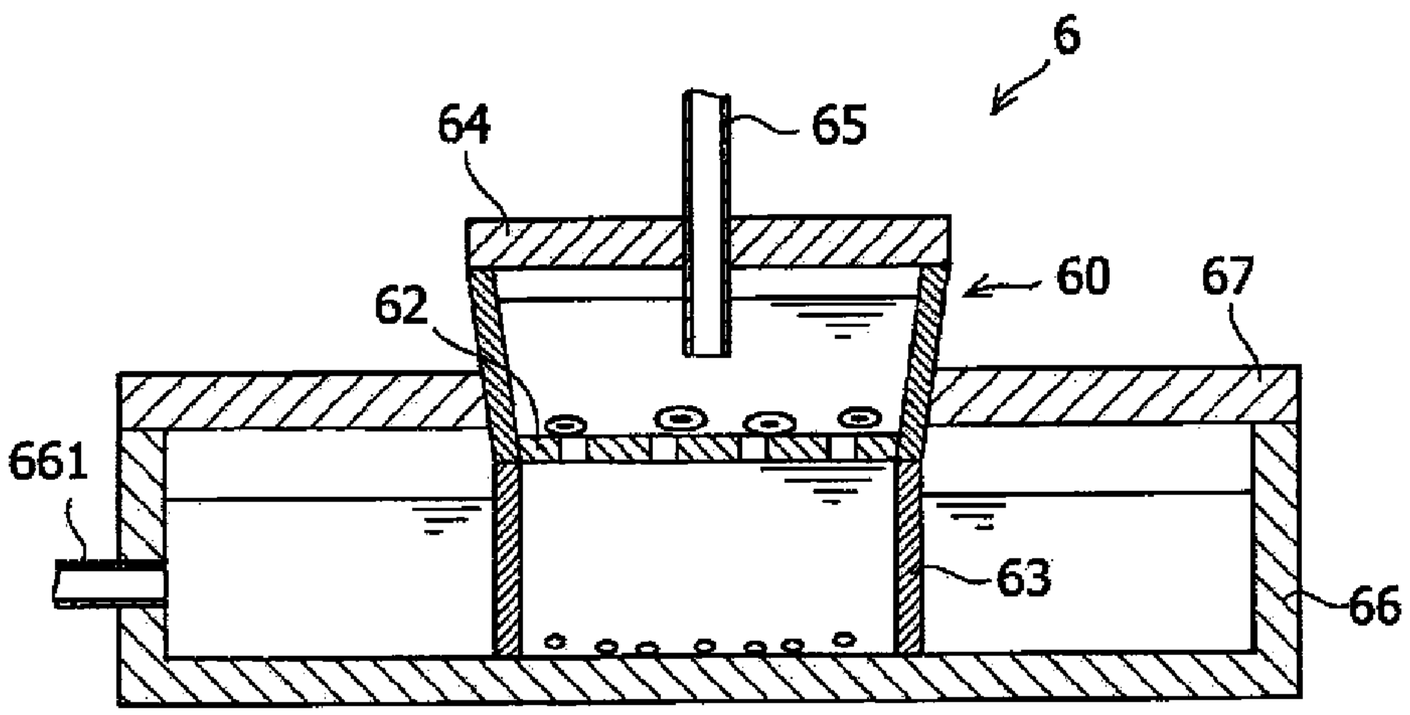

Fig. 22(b)

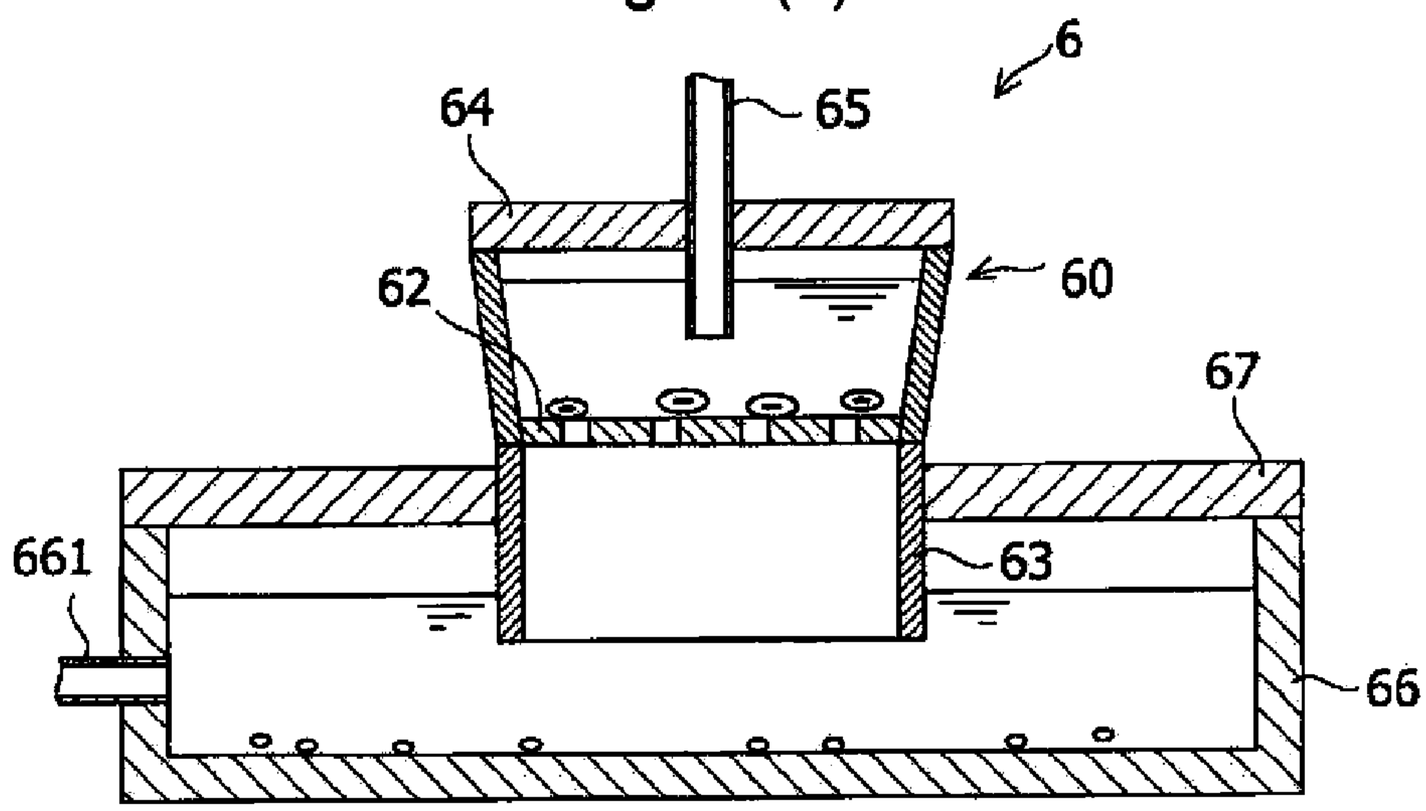

Fig. 23(a)

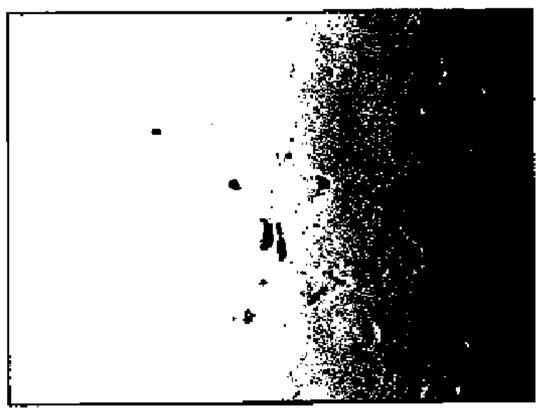

Fig. 23(b)

MEMBRANE-SEPARATION-TYPE CULTURE DEVICE, MEMBRANE-SEPARATION-TYPE CULTURE KIT, STEM CELL SEPARATION METHOD USING SAME, AND SEPARATION MEMBRANE

TECHNICAL FIELD

[0001] This disclosure relates to a membrane separation culture device and to a membrane separation culture kit which may be used to separate the stem cells and dental pulp stem cells of an organism of any species, and a method for separating stem cells using the same. In particular, the disclosure relates to a membrane separation culture device and a membrane separation culture kit used to separate dental pulp stem cells or mesenchymal stem cells with which the root canal is to be filled for regeneration of the dental pulp, and a method of separating stem cells using the same. In addition, the disclosure relates to a separation membrane, a surface modification method, and production of the separation membrane using the same.

BACKGROUND

[0002] At present, dental pulp stem cells used in biological root canal fillers for treatments such as treatment by extirpation of the pulp or the treatment of the infected root canal are fractions that are excellent in terms of angiogenic ability, nerve regeneration ability and dental pulp regeneration ability. Dental pulp-derived $CD31^{-}SP$ (side population) cells, $CD105^{+}$ cells, $CXCR4^{+}$ cells, or the like have been mainly used. SP cells are labeled with Hoechst33342, and a fraction that highly emits this pigment is then separated by flow cytometer using Hoechst Blue and Hoechst Red. Large quantities of stem cells are contained in this fraction. However, since Hoechst33342 is a DNA-binding pigment and it essentially requires the use of flow cytometer, it is said that Hoechst33342 is problematic in terms of the safety of cells.

[0003] On the other hand, in the case of using an antibody against a stem cell-specific membrane surface antigen, a method of using magnetic beads without using a flow cytometer has been developed. For example, as bone marrow stem cells, CD34 or CD133 antibody beads have been known. That method requires the use of considerable quantities of tissues or cells. Thus, if such tissues or cells are separated from dental pulp tissues, this method is inappropriate. In addition, in the case of human dental pulp, CD34-positive cells, and CD133-positive cells are hardly present in dental pulp test cells (0.01% and 0.5%, respectively) and, thus, the existing (commercially available) magnetic beads methods are not appropriate. Since CD105 or CXCR4 antibody beads must be prepared to order, they may be extremely expensive. Moreover, as a device for separating stem cells from adipose tissues, Celution 800/CRS system that is based on cell separation according to enzymatic digestion or centrifugation has already been used in clinical sites. However, that device requires large quantities of tissues, cells are obtained as a heterogeneous cell group containing large quantities of precursor cells, and it is also expensive. Furthermore, as a device for separating stem cells from bone marrow tissues, Bone Marrow MSC Separation Device has been commercialized. It is considered that the device is able to collect stem cells in a short time (20 minutes) by trapping bone marrow mesenchymal cells with fibers consisting of rayon and polyethylene. The device is relatively inexpensive, but it requires relatively large quantities of bone marrow tissues (spinal fluid) and the obtained cells are of a heterogeneous cell group containing large quantities of precursor cells. Hence, a device for separating stem cells from solid tissues without using enzymatic digestion and amplifying them has not yet been developed.

[0004] Cellculture Insert (Polycarbonate Membrane Transwell (registered trademark) Inserts; 2×10^{5} pores/cm^{2}, pore size: 8 μm, diameter of bottom surface: 6.4 mm, diameter of opening portion: 11.0 mm, height: 17.5 mm) (Corning) used as an upper structure, can be inserted into a 24-well plate (diameter: 15.0 mm, diameter of opening portion: 15.0 mm, height: 22.0 mm) (Falcon) used as a lower structure, and the thus prepared device can be used as a membrane separation device. However, since large quantities of cells adhere to a PET membrane or a polycarbonate membrane, migration of the cells to a lower layer cannot be carried out efficiently. Further, since that device has an open shape, it has a high risk of being contaminated by microorganisms, and thus the safety of cells cannot be guaranteed.

[0005] Accordingly, it is necessary to develop a method to inexpensively, safely and efficiently separate stem cells from human dental pulp tissues or human dental pulp cells.

[0006] To date, it has been known that $CXCR4^{+}$ cells, $C\text{-}MET^{+}$ cells, or $LIF\text{-}R^{+}$ cells present in the bone marrow, can be each concentrated to the stem cells of the bone marrow as a result of migration chemotaxis effect by utilizing the concentration gradient of their ligand SDF1, HGF, or LIF (NPL 1).

[0007] It has also been known that the stem cells of the bone marrow, peripheral blood and cord blood (hematopoietic stem cells of $CXCR4^{+}/lin^{-}/CD133^{+}/CD45^{+}$ cells, and mesenchymal stem cells of $CXCR4^{+}/lin^{-}/CD133^{+}/CD45^{-}$ cells) can also be concentrated by the concentration gradient of SDF1 in the same manner as described above (NPL 2). In that case, SDF-1 is placed in a lower chamber of a filter with a pore diameter (pore size) of 5 μm in a commercially available Costar Transwell 24-well, and cells to be concentrated are placed in an upper portion thereof. However, in view of safety, SDF-1 has not yet received pharmaceutical approval and, thus, it has been desired to develop a safe and effective migration factor that can be substituted for SDF-1.

[0008] Platelet-derived sphingosine-1-phosphate (S1P) has been known as a factor effective for migration of bone marrow stem cells (NPL 3), and protocatechuic acid has been known as a factor effective for migration of adipose stem cells (NPL 4). However, problems regarding safety have not yet been solved. Moreover, it has been known that dental pulp $CD31^{-}SP$ cells excellent in terms of angiogenic ability, nerve regeneration ability and dental pulp regeneration ability migrate towards SDF-1 or VEGF (NPL 5). However, at present, migration factors effective for separation of dental pulp stem cells excellent in terms of angiogenic ability, nerve regeneration ability and dental pulp regeneration ability have not been discovered, other than SDF-1.

[0009] A membrane separation culture device and a migration factor, which are capable of separating dental pulp stem cells safely and practically, are required. In addition, in biological studies, it has been desired to clarify a mechanism of inducing differentiation of stem cells into various types of tissues, not only in human beings but also in various organism species. With progression of regenerative medicine, it has been strongly desired to develop a membrane separation culture device and a migration factor, which are capable of separating stem cells simply and stably.

[0010] A medical separation membrane brought into contact with body fluid, blood or cells has many problems to be solved. For example, when proteins, platelets, or cells adhere to such a medical separation membrane, they cause a reduction in the performance of the separation membrane or vital reactions, and also promote adsorption of the cells. In addition, in the case of water-treating membranes used in water purifiers, the adhesion of proteins or organic substances causes a reduction in the performance of such a separation membrane. To solve such a problem, an attempt has been made to hydrophilize the separation membrane, and various studies have been conducted. For instance, a method which comprises mixing polyvinyl pyrrolidone as a hydrophilic polymer into a membrane consisting of polysulfone at the stage of the formation of the membrane and then molding the mixture to impart hydrophilicity to the membrane and to suppress contamination (Japanese Patent Publication No. 2-18695). However, to impart hydrophilicity to the surface of the membrane, that method is subjected to various restraints. For example, it is necessary to increase the amount of a hydrophilic polymer used in a stock solution for membrane formation, the hydrophilic polymer is limited to that compatible with a polymer used as a base material, and the optimal composition of a stock solution should be determined depending on the intended use of the material.

[0011] In addition, Japanese Patent Laid-Open No. 8-131791 discloses a method of coating a membrane with polyvinyl acetal diethylamino acetate and a hydrophilizing agent to hydrophilize the membrane. In that method, there is a concern that polyvinyl acetal diethylamino acetate may cover the hydrophilizing agent and effects regarding non-adhesion may be significantly reduced. Moreover, there is another concern that the separation performance of a membrane may be reduced because the membrane is immersed both in a solution of the polyvinyl acetal diethylamino acetate and in a solution of the hydrophilizing agent.

[0012] There are disclosed a method which comprises water-insolubilizing a hydrophilic component such as polyvinyl pyrrolidone, with a high-energy beam, and then introducing the resulting hydrophilic component into the formed membrane (Japanese Patent Publication No. 8-9668), and a method which comprises allowing a polysulfone separation membrane to come into contact with a solution of a hydrophilic polymer such as polyvinyl pyrrolidone, and then forming an insolubilized coating layer by radiation crosslinking (Japanese Patent Laid-Open No. 6-238139). However, those methods have been problematic in that, since such an aqueous polymer such as polyvinyl pyrrolidone and a polysulfone polymer have a low intermolecular interaction, it is difficult to form a coating layer.

[0013] To solve the aforementioned problem, there has been disclosed a method which comprises allowing a polyvinyl alcohol aqueous solution having a certain range of saponification degree to come into contact with a polysulfone separation membrane to efficiently form a coating layer on the surface of the membrane as a result of a hydrophobic interaction between polysulfone and vinyl acetate (Japanese Patent Laid-Open No. 2006-198611). However, that publication does not describe a method of suppressing adhesion. Thus, as a result of studies we conducted, we found that, if a separation membrane is simply coated with polyvinyl alcohol, the performance of the separation membrane is significantly reduced. Also, it has been known that a hydroxyl group of polyvinyl alcohol tends to activate a complement when it comes into contact with blood.

[0014] Furthermore, it is said that even if the surface of a material is coated with a hydrophilic polymer such as polyvinyl pyrrolidone or polyethylene glycol, the effect of suppressing adhesion can be obtained only temporarily (NPL 6). That is to say, a separation membrane module, in which blood compatibility is satisfied with a high-performance separation membrane, has not yet been established.

[0015] Conventional flow cytometry and antibody-coated magnetic beads method cannot be safe, highly-efficient and inexpensive separation methods, which satisfy standards used in clinical sites (Good Manufacturing Practice (GMP)). Thus, it could be helpful to provide: a culture device capable of obtaining desired stem cells even from small quantities of tissues safely, highly efficiently and inexpensively, without using the conventional flow cytometry or antibody-coated magnetic beads in dental pulp regenerative medicine and other regenerative medicines; a migration factor used in the culture device; and a method for separating stem cells. Further, it could be helpful to provide a membrane separation culture device, a membrane separation culture kit, and a method of separating stem cells which can be applied to separation of stem cells of all organism species (embryonic stem cells, iPS cells, and tissue stem cells).

[0016] It could further be helpful to provide a high-performance separation membrane, an improved separation membrane which has compactness sufficient to be used in ordinary cell culture incubators or clean benches, and in which not only filtration performance caused by pore diameter but also surface affinity has been improved.

SUMMARY

[0017] We found that stem cells are allowed to selectively pass from the upper portion of a membrane to a lower portion thereof using the concentration gradient of a cell migration factor so that the stem cells can be separated. Thus, we provide a membrane separation culture device comprising: an upper structure constituted with a vessel in which at least a portion of the bottom thereof is formed with a separation membrane having pores that allow stem cells to permeate therethrough; and a lower structure constituted with a vessel that retains a fluid in which the membrane of the upper structure is immersed.

[0018] It is preferable that the separation membrane comprise: a base material membrane consisting of a hydrophobic polymer; and a functional layer formed by allowing one or more hydrophilic polymers selected from a vinyl pyrrolidone polymer, a polyethylene glycol polymer and a vinyl alcohol polymer to bind to the surface of the base material membrane via a covalent bond; wherein the weight percentage of the hydrophilic polymer(s) constituting the functional layer is 1.5% to 35% based on the total weight of the separation membrane.

[0019] It is preferable that the size of the pore be 3 μm to 10 μm and the density be 1×10^5 to 4×10^6 pores/cm^2.

[0020] It is preferable that the membrane separation culture device comprise a plurality of the upper structures, and further comprise a frame body that is accommodated in the lower structure and comprises a plate-like member in which a plurality of holes are each established to lock the plurality of the upper structures.

[0021] Alternatively, it is preferable that the membrane separation culture device comprise a plurality of the upper

structures, and further comprise a frame body that is accommodated in the lower structure and comprises a plate-like member in which a plurality of holes are each established to lock the plurality of the upper structures, and that the lower structure be constituted with a plurality of vessels each corresponding to the plurality of the upper structures.

[0022] Alternatively, it is also preferable that the plurality of the upper structures have membranes each having a different pore size and/or a different pore density.

[0023] It is preferable that the membrane separation culture device further comprise a lid structure that covers or hermetically seals the upper structure and the lower structure.

[0024] It is preferable that the lid structure further comprises a gas exchanger comprising a gas inlet port and a gas discharge port.

[0025] It is preferable that at least a portion of the lid structure be formed with a membrane having pores whose pore size is 1 to 100 nm.

[0026] It is preferable that a hermetic sealing elastic body be established between the lid structure and the lower structure.

[0027] It is preferable that the membrane separation culture device further comprise a temperature control system containing a temperature-measuring device and a temperature-controlling device.

[0028] It is preferable that the lower structure further comprise a medium replacement system comprising a medium inlet port and a medium outlet port.

[0029] We also provide a membrane separation culture kit comprising the membrane separation culture device according to any of the descriptions and cell migration factor(s) to be poured into the lower structure.

[0030] It is preferable that the cell migration factor(s) be one or more selected from SDF-1, G-CSF, bFGF, TGF-β, NGF, PDGF, BDNF, GDNF, EGF, VEGF, SCF, MMP3, Slit, GM-CSF, LIF, HGF, SIP, protocatechuic acid, and serum.

[0031] It is preferable that the concentration of the cell migration factor(s) be 1 ng/ml to 500 ng/ml.

[0032] It is preferable that the kit further comprise serum to be poured into the lower structure, and that the cell migration factor is G-CSF or bFGF.

[0033] We still further provide a method of separating stem cells using the membrane separation culture device, wherein the method comprises: a step of dispersing test cells or test tissues on the membrane of the upper structure; a step of filling the vessel as a lower structure with a medium containing cell migration factor(s); and a step of allowing the membrane of the upper structure to come into contact with the medium in the lower structure.

[0034] It is preferable that the cell migration factor(s) is one or more selected from SDF-1, G-CSF, bFGF, TGF-β, NGF, PDGF, BDNF, GDNF, EGF, VEGF, SCF, MMP3, Slit, GM-CSF, LIF, HGF, SIP, protocatechuic acid, and serum.

[0035] It is preferable that the concentration of the cell migration factor(s) is 1 ng/ml to 500 ng/ml.

[0036] It is preferable that the test cells are dispersed at a density of 3×10^2 cells to 3×10^4 cells per mm^2 of the separation membrane.

[0037] We still further provide a method of separating stem cells, wherein the stem cells are dental pulp stem cells, the cell migration factor is G-CSF or bFGF, the concentration of the G-CSF or bFGF is 50 to 150 ng/ml, the test cells are dispersed at a density of 3×10^2 to 1.5×10^3 cells per mm^2 of the separation membrane, or the test tissues are left at rest at a density of 0.1 mg to 1 mg per mm^2 of the separation membrane, and serum is added to a medium containing the cell migration factor at a volume percentage of 5% to 20% based on the volume of the medium.

[0038] We yet further provide a method of separating stem cells, wherein the stem cells are bone marrow stem cells or adipose stem cells, the cell migration factor is G-CSF or bFGF, the concentration of the G-CSF or bFGF is 50 to 150 ng/ml, the test cells are dispersed at a density of 3×10^2 to 1.5×10^3 cells per mm^2 of the separation membrane, or the test tissues are left at rest at a density of 0.1 mg to 1 mg per mm^2 of the separation membrane, and serum is added to a medium containing the cell migration factor at a volume percentage of 5% to 20% based on the volume of the medium.

[0039] We also found that the separation membrane and separation membrane module, which are excellent in terms of blood compatibility and have small amounts of proteins or organic substances adhering thereto, can be achieved with the following configurations.

[0040] Specifically, we provide a separation membrane comprising: a base material membrane consisting of a hydrophobic polymer; and a functional layer formed by allowing one or more hydrophilic polymers selected from a vinyl pyrrolidone polymer, a polyethylene glycol polymer and a vinyl alcohol polymer to bind to the surface of the base material membrane via a covalent bond; wherein the weight percentage of the hydrophilic polymer(s) constituting the functional layer is 1.5% to 35% based on the total weight of the separation membrane.

[0041] It is preferable that the base material membrane has pores with a pore size of 1 to 10 μm and be used for cell separation.

[0042] It is preferable that the hydrophobic polymer be selected from the group consisting of a sulfone polymer an amide polymer, a carbonate polymer, an ester polymer a urethane polymer, an olefin polymer, and an imide polymer.

[0043] It is preferable that the separation membrane according to any of the descriptions be used for separating cells by permeation.

[0044] Further, we provide a method of producing the separation membrane which comprises: an immersion step of immersing a base material membrane consisting of a hydrophobic polymer having a water absorption percentage of 2% or less in a treating aqueous solution containing one or more hydrophilic polymers selected from a vinyl pyrrolidone polymer, a polyethylene glycol polymer and a vinyl alcohol polymer at a concentration of 10 to 2000 ppm, and also containing a 0.01% to 0.2% alcohol; and a modification step of irradiating the base material membrane with a high-energy beam to modify the surface of the membrane to have a protein adhesion-suppressing property and a cell adhesion-suppressing property.

[0045] We further provide a method of producing the separation membrane which comprises: an immersion step of immersing a base material membrane consisting of a hydrophobic polymer having a water absorption percentage of more than 2% in a treating aqueous solution containing one or more hydrophilic polymers selected from a vinyl pyrrolidone polymer, a polyethylene glycol polymer and a vinyl alcohol polymer at a concentration of 10 to 2,000 ppm; and a modification step of irradiating the base material membrane with a high-energy beam to modify the surface of the membrane to have a protein adhesion-suppressing property and a cell adhesion-suppressing property.

[0046] It is preferable that the hydrophobic polymer be selected from the group consisting of a sulfone polymer, an amide polymer, a carbonate polymer, an ester polymer, a urethane polymer, an olefin polymer, and an imide polymer.

[0047] We still further provide a method of modifying the surface of a molded body, which comprises: an immersion step of immersing a molded body having a water absorption percentage of 2% or less in a treating aqueous solution containing one or more hydrophilic polymers selected from a vinyl pyrrolidone polymer, a polyethylene glycol polymer and a vinyl alcohol polymer at a concentration of 10 to 2,000 ppm, and also containing a 0.01% to 0.2% alcohol; and a modification step of irradiating the molded body with a high-energy beam to modify the surface of the molded body to have a protein adhesion-suppressing property and a cell adhesion-suppressing property.

[0048] We yet further provide a method of modifying the surface of a molded body, which comprises: an immersion step of immersing a molded body having a water absorption percentage of more than 2% in a treating aqueous solution containing one or more hydrophilic polymers selected from a vinyl pyrrolidone polymer, a polyethylene glycol polymer and a vinyl alcohol polymer at a concentration of 10 to 2000 ppm; and a modification step of irradiating the molded body with a high-energy beam to modify the surface of the molded body to have a protein adhesion-suppressing property and a cell adhesion-suppressing property.

[0049] Also, we provide a method of producing the separation membrane using the methods of modifying a molded body.

[0050] Stem cells can thus be separated even from small quantities of cells safety, highly efficiently and inexpensively, using a membrane separation culture device and cell migration factor(s). In addition, permeation pores with a suitable size are selected depending on the size of cells so that the membrane separation culture device can be used to separate the stem cells of all organism species. Otherwise, by selecting suitable cell migration factor(s), the membrane separation culture device can be applied to separation of all types of stem cells including embryonic stem cells, iPS cells, and tissue stem cells. Moreover, by changing the number of separation membranes in membrane separation culture, the membrane separation culture device can also be applied to various amounts of tissues or cells. By adopting completely-sealed-type upper structure and lower structure, it becomes possible to separate stem cells, which comply with GMP and can be practically used in clinical sites. Furthermore, the membrane separation culture device can be broadly used for both experimental use and clinical use, and greatly contributes to the development of regenerative medicine. Membrane-separated cells are further advantageous in that, in particular, stem cells separated by a membrane from middle-aged and elderly people have a small level of phenotypical change associated with amplification. Since such stem cells hardly become senescent and are hardly aged together with amplification, they can be effectively functional in clinical use. Moreover, a membrane separation device is further advantageous in that stem cells can be separated not only from cells but also from tissues, without previously dispersing the cells by enzymatic digestion or the like, which leads to a reduction in the time required for such enzymatic digestion and the guarantee of safety by nonuse of enzymes.

[0051] Furthermore, the separation membrane suppresses a decrease in separation efficiency caused by adhesion of cells during cell separation, and the separation membrane is characterized in that a polymer is localized on the surface of the functional layer of the separation membrane. The separation membrane can be preferably used to suppress protein or cell adhesion on the surface of a separation membrane that has been molded without mixing a hydrophilic polymer into a stock solution for membrane formation, for example, on the surface of a membrane on which pores have been formed by irradiation of an electron beam.

BRIEF DESCRIPTION OF THE DRAWINGS

[0052] FIG. **1** is a schematic view showing a membrane separation culture device according to a first example.

[0053] FIG. **2** is a schematic view showing a membrane separation culture device according to a second example.

[0054] FIG. **3** is a schematic view showing a configuration of a membrane separation culture device according to a third example.

[0055] FIG. **4** is a schematic view showing a configuration of a membrane separation culture device according to a fourth example.

[0056] FIG. **5** is a schematic view showing a configuration of a membrane separation culture device according to a fifth example.

[0057] FIG. **6**(*a*) is a view showing differences in the migration of dog CD105-positive cells depending on the types of cell migration factors, analyzed by TaxiScan, and time course; and FIG. **6**(*b*) is a view showing differences in the migration of dog CD105-positive cells depending on various concentrations of the cell migration factor G-CSF, analyzed by TaxiScan.

[0058] FIG. **7**(*a*) is a view showing differences in the migration of human dental pulp test cells depending on a change in fetal bovine serum concentrations, analyzed by TaxiScan, and time course; and FIG. **7**(*b*) is a view showing differences in the migration of human dental pulp test cells by the cell migration factors G-CSF and SDF-1 (final concentration: 100 ng/ml) in comparison with fetal bovine serum, analyzed by TaxiScan.

[0059] FIG. **8**(*a*) is a view showing dental pulp stem cells obtained by dispersing fresh dog primary dental pulp cells (1×10^5 cells/250 μl) in an upper portion of a separation membrane by use of our membrane separation culture device (2×10^5 pores/cm^2, pore size: 3 μm), placing 100 ng/ml G-CSF in DMEM containing 10% dog serum in a lower structure of the separation membrane, then, 6 hours later, replacing the medium with fresh medium, then removing the G-CSF, and then further performing a culture for 1 day; FIG. **8**(*b*) is a view showing dental pulp stem cells obtained by dispersing fresh dog primary dental pulp cells (1×10^5 cells/250 μl) in an upper portion of a separation membrane by use of our membrane separation culture device (2×10^5 pores/cm^2, pore size: 3 μm), placing 100 ng/ml G-CSF in DMEM containing 10% dog serum in a lower structure of the separation membrane, then, 6 hours later, replacing the medium with fresh medium, then removing the G-CSF, and then further performing a culture for 7 days; and FIG. **8**(*c*) is a view showing dental pulp stem cells obtained by dispersing fresh dog primary dental pulp cells (1×10^5 cells/250 μl) in an upper portion of a separation membrane by use of our membrane separation culture device (2×10^5 pores/cm^2, pore size: 3 μm), placing 100 ng/ml SDF-1 in DMEM containing 10% dog serum in a lower structure of the separation membrane, then, 6 hours later, replacing the

medium with fresh medium, then removing the SDF-1, and then further performing a culture for 1 day.

[0060] FIG. **9**(*a*) is a view showing the angiogenesis-inducing ability of the 5th-generation dental pulp stem cells in a test tube which were separated and cultured using our membrane separation culture device and G-CSF; and FIG. **9**(*b*) is a view showing the neurosphere-forming ability of the 5th-generation dental pulp stem cells in a test tube which were separated and cultured using our membrane separation culture device and G-CSF.

[0061] FIG. **10**(*a*) is a view showing regeneration of dental pulp observed 14 days after transplantation of dental pulp stem cells, which had been separated and cultured using our membrane separation culture device and G-CSF, into the root canal after extirpation of dog pulp;

[0062] FIG. **10**(*b*) is a high magnification view of the site shown in B of FIG. **10**(*a*); FIG. **10**(*c*) is a high magnification view of the site shown in C of FIG. **10**(*a*), which shows odontoblasts differentiated and aligned along the dentin side wall of the regenerated dental pulp in the root canal; and FIG. **10**(*d*) is a view showing a normal dental pulp of a dog of the same age at the same site.

[0063] FIG. **11** is a view showing human dental pulp stem cells obtained by dispersing fresh human primary dental pulp cells (1×10^5 cells/250 μl) in an upper portion of a separation membrane by use of our membrane separation culture device (2×10^5 pores/cm^2, pore size: 3 μm), placing 100 ng/ml G-CSF in DMEM containing 10% human serum in a lower structure of the separation membrane, then, 6 hours later, replacing the medium with fresh medium, then removing the G-CSF, and then further performing a culture for 8 days.

[0064] FIG. **12**(*a*) is a view showing the separated human dental pulp stem cells obtained by dispersing fresh human primary dental pulp cells (1×10^5 cells/100 μl) in an upper portion of a separation membrane by use of our membrane separation culture device (1×10^5 pores/cm^2, pore size: 8 μm), placing DMEM containing 10% human serum in a lower structure of the separation membrane, then, 22 hours later, replacing the medium with fresh medium, and then further performing a culture for 3 days; FIG. **12**(*b*) is a view showing human dental pulp stem cells obtained by dispersing fresh human primary dental pulp cells (1×10^5 cells/100 μl) in an upper portion of a separation membrane by use of the same membrane separation culture device as mentioned above, placing 10 ng/ml G-CSF in DMEM containing 10% human serum in a lower structure of the separation membrane, then, 22 hours later, replacing the medium with fresh medium, then removing the G-CSF, and then further performing a culture for 3 days; FIG. **12**(*c*) is a view showing human dental pulp stem cells obtained by dispersing fresh human primary dental pulp cells (1×10^5 cells/100 μl) in an upper portion of a separation membrane by use of the same membrane separation culture device as mentioned above, placing 100 ng/ml G-CSF in DMEM containing 10% human serum in a lower structure of the separation membrane, then, 22 hours later, replacing the medium with fresh medium, then removing the G-CSF, and then further performing a culture for 3 days; FIG. **12**(*d*) is a view showing human dental pulp stem cells observed 7 days after completion of the culture of 10% human serum described in FIG. **12**(*a*) above; and FIG. **12**(*e*) is a view showing human dental pulp stem cells observed 7 days after completion of the culture of 100 ng/ml G-CSF described in FIG. **12**(*c*) above.

[0065] FIG. **13**(*a*) is a view showing pig dental pulp stem cells obtained by dispersing fresh pig primary dental pulp cells (1×10^5 cells/100 μl) in an upper portion of a separation membrane by use of the membrane separation culture device (1×10^5 pores/cm^2, pore size: 8 μm), placing 100 ng/ml G-CSF in DMEM containing 10% fetal bovine serum in a lower structure of the separation membrane, then, 22 hours later, replacing the medium with fresh medium, then removing the G-CSF, and then further performing a culture for 3 days; FIG. **13**(*b*) is a view showing pig bone marrow stem cells obtained by dispersing fresh pig primary bone marrow cells, instead of fresh pig primary dental pulp cells, then performing membrane separation, and then performing a culture for 3 days; FIG. **13**(*c*) is a view showing pig adipose stem cells obtained by dispersing fresh pig primary adipose cells, instead of fresh pig primary dental pulp cells described in FIG. **13**(*a*) above, and then performing a culture for 3 days; and FIG. **13**(*d*) is a view showing pig dental pulp stem cells obtained by dispersing the fresh pig primary dental pulp cells described in FIG. **13**(*a*) above, then performing membrane separation, and then performing a culture for 8 days.

[0066] FIG. **14** is a graph showing a comparison regarding the migration ability of unseparated human dental pulp test cells depending on various types of migration factors.

[0067] FIG. **15** is a graph showing a comparison regarding the migration ability of human dental pulp test cells depending on the formulated migration factors and serum.

[0068] FIG. **16** is a graph showing a comparison regarding the cell proliferative ability of membrane-separated dental pulp cells that had been separated using various concentrations of G-CSF, wherein human serum was used.

[0069] FIG. **17** is a graph showing a comparison regarding the cell proliferative ability of membrane-separated dental pulp cells that had been separated using various concentrations of G-CSF, wherein 100 ng/ml G-CSF was used.

[0070] FIG. **18** is a graph showing a comparison regarding the cell migration ability of membrane-separated dental pulp cells to G-CSF that had been separated using various concentrations of G-CSF.

[0071] FIG. **19** is a graph showing a comparison regarding cell proliferative ability to fetal bovine serum, which was made among membrane-separated dental pulp, bone marrow and adipose cells that had been each separated using 100 ng/ml G-CSF, and test cells.

[0072] FIG. **20** is a graph showing a comparison regarding cell proliferative ability to 100 ng/ml G-CSF, which was made among membrane-separated dental pulp, bone marrow and adipose cells that had been each separated using 100 ng/ml G-CSF, and test cells.

[0073] FIG. **21** is a graph showing a comparison regarding migration ability to G-CSF, which was made among membrane-separated dental pulp, bone marrow and adipose cells that had been each separated using 100 ng/ml G-CSF, and test cells.

[0074] FIG. **22** is a schematic view showing a configuration of a membrane separation culture device according to a sixth example.

[0075] FIG. **23**(*a*) is a view showing dental pulp stem cells obtained by leaving at rest minced fresh dog dental pulp tissues (2 mg/200 μl) on an upper portion of a membrane by use of our membrane separation culture device (1×10^5 pores/cm^2, pore size: 8 μm), then placing 100 ng/ml G-CSF in DMEM containing 10% serum in a lower structure of the membrane, and then leaving them for 24 hours; and FIG.

23(*b*) is a view showing adipose stem cells, 24 hours after being obtained in the same manner as in FIG. **23**(*a*) above by leaving at rest minced fresh dog adipose tissues (2 mg/200 μl) on an upper portion of a membrane, then placing 100 ng/ml G-CSF in DMEM containing 10% serum in a lower structure of the membrane, and then leaving them for 24 hours.

REFERENCE SIGNS LIST

[0076] **1**, **2**, **3**, **4**, **5** Membrane separation culture device
[0077] **10**, **20**, **30**, **40**, **50**, **60** Upper structure
[0078] **12**, **22**, **32**, **62** Separation membrane
[0079] **121**, **221** Pore
[0080] **13**, **23**, **33**, **43**, **53**, **63** Lower structure
[0081] **231** Medium inlet port
[0082] **232** Medium outlet port
[0083] **24**, **34**, **44** Lid structure
[0084] **241** Gas inlet port
[0085] **242** Gas discharge port
[0086] **331**, **431** Elastic body
[0087] **35**, **335** Retention mechanism
[0088] **45**, **55** Frame body
[0089] **451**, **551** Hole
[0090] **452**, **552** Partition
[0091] **453**, **553** Retention mechanism
[0092] **64** Lid structure
[0093] **65** Introduction port
[0094] **66** Dish
[0095] **67** Lid structure
[0096] **661** Medium recovery port
[0097] **100** Medium
[0098] **200** Test cells
[0099] **300** Medium
[0100] a Inert gas
[0101] b Emission gas
[0102] c Medium
[0103] d Used medium

DETAILED DESCRIPTION

[0104] We provide a separation membrane in which adhesion of cells to the membrane upon separation of the cells by permeation is suppressed by hydrophilizing a membrane consisting of a hydrophobic polymer without impairing separation performance. Accordingly, the membranes are preferably used in the fields of separation and purification of cells including blood purification field or regenerative medicine as typical examples. Moreover, by such a polymer surface modification method, only the surface of a polymer can be simply modified, and sterilization can be simultaneously carried out. Hence, when compared to conventional methods, the polymer surface modification method can contribute to production efficiency.

[0105] Hereinafter, our devices, kits, membranes and methods will be described in detail, while referring to the figures. However, the following explanation is not intended to limit the scope of this disclosure.

First Example

[0106] A membrane separation culture device **1** according to a first example is composed of an upper structure **10** and a lower structure **13**. The upper structure **10** is configured to contain a medium **100** containing test cells **200** or test tissues and to retain the test cells **200** or test tissues on a separation membrane **12**. On the other hand, the lower structure **13** is configured to contain a medium **300** containing migration factor(s) (not shown in the figure) and receive migrating stem cells.

[0107] The upper structure **10** is a vessel consisting of a lateral surface portion **11** and a round-shaped bottom surface portion, wherein the bottom surface portion is formed with the separation membrane **12** having a plurality of pores **121**. The type of the upper structure **10** is not particularly limited, as long as it can contain the medium **100** and the test cells **200** or the test tissues therein. The term "test cells" is used to mean cells which have been released from intercellular adhesion in tissues by enzyme treatment and have not yet been separated, or cells which have been subcultured and dispersed. For example, the vessel is preferably capable of containing approximately 100 to 250 μl of the medium **100** and the test cells **200**. The term "test tissues" is used herein to mean tissues which have been minced, but have not been digested with enzyme and have not been dispersed.

[0108] The separation membrane **12** constituting the bottom surface of the upper structure **10** has a plurality of pores **121** for allowing stem cells to pass therethrough. The pore size is 1 μm to 100 μm, preferably 3 μm to 10 μm, and more preferably 5 μm to 8 μm. This is because stem cells are allowed to permeate through the pores. In addition, the pore density is 2.5×10^3 to 2.5×10^7 pores/cm^2, and preferably 1×10^5 to 4×10^6 pores/cm^2. To allow stem cells to efficiently pass through the pores, the higher the porosity rate, the better results that can be obtained.

[0109] As a material for the separation membrane **12**, it is preferable to use a material comprising, as a base material, a hydrophobic polymer such as PET, polycarbonate, polysulfone, polypropylene, polyvinylidene fluoride or polyamide. Moreover, the thickness of the separation membrane **12** is set at preferably 10 to 100 μm, and more preferably 10 to 25 μm. This is because the surface of stem cells is not damaged when the stem cells migrate, and in particular, when the stem cells are allowed to pass through pores.

[0110] For the separation membrane **12** to have a non-cell-adhesive property, the surface of the separation membrane is preferably coated with a coating agent. In particular, such a coating agent may be applied to an inner surface of the upper structure **10**, which is a surface allowed to come into contact with the test cells **200** or test tissues, when the test cells **200** or test tissues are dispersed. Examples of the coating agent that can be used herein include known non-cell-adhesive coating agents such as an ethyleneoxide/propyleneoxide copolymer (trade name: Pluronic F108, ADEKA CORPORATION), coating agents in which poly2-hydroxyethyl methacrylate is dissolved in 95% ethanol to a concentration of 5 mg/ml (Folkman J & Moscona A, Nature 273: 345-349, 1978, Japanese Patent Application Laid-Open No. 8-9966 and the like), and a branched polyalkylene glycol derivative (WO2009/072590), but the examples are not limited thereto. Any given non-cell-adhesive coating agents can be used. The coating thickness is not particularly limited, as long as it is in a range necessary for imparting a sufficient non-cell-adhesive property to a base material membrane such as PET, polycarbonate or polyvinylidene fluoride. Thus, for example, the thickness of the coating agent is set at preferably 10 to 100 μm, and more preferably 10 to 25 μm, although it depends on the type of the coating agent.

[0111] A particularly preferred method of modifying the separation membrane **12** will be further described. In the above-described methods using coating agents, the remaining

organic solvent may have adverse effects on cells such that the pores of the separation membrane may be clogged with the remaining organic solvent, or elution may occur with an aqueous culture medium. Accordingly, the surface of the separation membrane is preferably modified by the following covalent bond method.

[0112] That is to say, a base material membrane consisting of a hydrophobic polymer, on which pores with a desired pore diameter have been formed at a high porosity rate, is immersed in a treating aqueous solution comprising a vinyl pyrrolidone polymer, an ethylene glycol polymer and/or a vinyl alcohol polymer, and as necessary, an alcohol, and thereafter, the base material membrane is irradiated with a high-energy beam to perform surface modification, thereby producing a separation membrane.

[0113] The polyvinyl pyrrolidone polymer is a polymer selected from the group consisting of polyvinyl pyrrolidone, a vinyl pyrrolidone/vinyl acetate copolymer, a vinyl pyrrolidone/vinyl alcohol copolymer, a vinyl pyrrolidone/styrene copolymer, a vinyl pyrrolidone/dimethyl aminoethyl methacrylate copolymer, and a modified polymer thereof. The ethylene glycol polymer includes those containing an ester group on the side chain thereof. As vinyl alcohol polymers, various types of polymers can be obtained depending on saponification degree. However, the type of such a vinyl alcohol polymer is not limited. These membrane surface-modifying polymers are preferably water-soluble polymers. Thus, polymers having a number average molecular weight of 10,000 to 1,000,000 can be used, for example. However, as long as the polymer is water-soluble, its molecular weight is not limited to the aforementioned molecular weight. The concentration of a polypyrrolidone polymer in the aforementioned treating aqueous solution is preferably 10 to 5000 ppm. If the concentration of the polymer becomes high, pores may be clogged with the treating aqueous solution. Accordingly, the concentration of the polypyrrolidone polymer is more preferably 10 to 2000 ppm.

[0114] Moreover, to efficiently modify the surface of the base material membrane, when the base material membrane has a water absorption percentage of 2% or less, it is preferable to further add an alcohol to the treating aqueous solution. It is to be noted that the water absorption percentage of the base material membrane is defined as a weight increase percentage obtained by immersing a base material membrane having a thickness of 100 μm or less in water at 23° C. for 24 hours and then measuring the weight increased.

[0115] If taking into consideration safety when an alcohol remains, the alcohol that is added when the water absorption percentage of the base material membrane is 2% or less is preferably ethanol. However, examples of an alcohol added are not limited thereto. The concentration of the alcohol added is preferably 1% by weight or less, and for safety, it is more preferably 0.5% by weight or less, and further preferably 0.1% by weight or less, based on the weight of the treating aqueous solution.

[0116] As a high-energy beam, any of UV, an electron beam, and a γ-ray can be used. Among them, an electron beam or a γ-ray is more preferable because these easily enhance a reaction rate. The dose to be applied is preferably 5 to 35 kGy. It is also possible to simultaneously carry out surface modification and sterilization by irradiating, in particular, the entire culture device, with a dose of, for example, approximately 25 kGy, which is considered to be a dose used for sterilization.

[0117] The thus obtained separation membrane is useful from the viewpoint of non-cell-adhesive property and, thus, it can be effectively used in the membrane separation device according to this example.

[0118] As a material for the lateral surface portion **11** of the upper structure **10**, an ordinary material that is generally used as a cell culture device can be used, and it may be made of plastic such as polyethylene terephthalate (PET), polystyrene, polypropylene (PP) or polycarbonate.

[0119] The dimension of the upper structure **10** is not limited to a specific size. For example, the diameter of the bottom surface portion may be set at 5 to 8 mm. The diameter of an opening portion of the vessel, which is specified with the top edge of the lateral surface portion **11**, may be set at 6 to 10 mm, for example. The height from the bottom surface portion **12** to the opening portion, namely, the depth of the vessel may be set at 10 to 15 mm, for example. It is to be noted that these values are given only for illustrative purpose, and that the dimension of the vessel constituting the upper structure **10** can be determined by a person skilled in the art, as appropriate, depending on purposes such as the type of test cells or test tissues, or the type of stem cells to be collected.

[0120] Next, the lower structure **13** is a vessel consisting of a bottom surface portion and a lateral surface portion, and it has an opening portion on an upper surface. With regard to a material for the lower structure **13**, the same materials can be used for both the lateral surface and the bottom surface thereof. It is preferable to use polystyrene, glass, and the like. This is because these materials impart a cell adhesion property and a cell proliferation property to the lower structure. It is to be noted that it is not always necessary that the bottom surface portion be fixed and integrated with the lateral surface portion in the lower structure, but that it is also possible to form the bottom surface portion of the lower structure with a material different from the material of the lower structure depending on examples.

[0121] The lower structure **13** can be detachably equipped with the upper structure **10**. When the upper structure **10** is equipped into the lower structure **13**, the separation membrane **12** of the vessel constituting the upper structure **10** is stored in the lower structure **13**. This is because a medium contained in the lower structure **13** is allowed to come into contact with the separation membrane **12** constituting the upper structure **10** in the below-mentioned method for separating stem cells so that it enables the passage of stem cells through the separation membrane. In the example shown in FIG. **1**, the separation membrane **12** is not allowed to come into contact with the bottom surface of the lower structure **13**, and a space is formed between the separation membrane **12** and the bottom surface portion of the lower structure **13**. This space can be filled with a medium.

[0122] To fix the positional relationship between the lower structure **13** and the upper structure **10** when the lower structure is equipped with the upper structure, the membrane separation culture device may also have a retention mechanism that is not shown in the figure. The retention mechanism may be established on either one of the upper structure **10** and the lower structure **13**, or may also be established on both of the two structures. The retention mechanism may be, for example, a flange or an unguiform member that extends from the top edge or a predetermined position of the lateral surface of the upper structure **10** towards the outside of the opening portion. Such a retention mechanism is allowed to come into contact with the top edge of the lateral surface of the lower

structure **13** so that it can retain the upper structure **10** at a predetermined height. Another example of the retention mechanism may be a pedate member, which is established on the upper structure **10** and extends downward from the bottom surface of the upper structure **10**. Such a retention mechanism is allowed to come into contact with the bottom surface of the lower structure **13** so that it can retain the upper structure **10** at a predetermined height. At this time, it is also possible to establish a member, which is fitted with the above described pedate member to fix it, also on the bottom surface of the lower structure.

[0123] In relation to the dimension of the above described upper structure **10**, as an example of the dimension of the lower structure **13**, the diameter of the bottom surface portion may be set at 7 to 15 mm, for example. In addition, the diameter of the opening portion of the vessel, which is specified with the top edge of the lateral surface portion of the lower structure **13**, may also be set to the aforementioned size. The depth of the lower structure **13** is preferably greater than the depth of the upper structure **10**, and it may be set at 11 to 20 mm, for example.

[0124] In this example, an upper structure comprising a bottom surface and an opening portion each having a round shape, wherein the diameter of the bottom surface is smaller than the diameter of the opening portion, is described as an example. However, the shape of the bottom surface portion **12** is not limited to a circle, and the bottom surface portion can also have an elliptical, square, polygonal or any given shape. Moreover, the relationship between the dimension of the bottom surface portion and the dimension of the opening portion is not limited to that as defined in this example. The dimension of the bottom surface portion may be identical to the dimension of the opening portion. Furthermore, the upper structure may also be configured to contain a partial surface of a sphere in which the bottom surface is consecutive with the lateral surface, as far as it has a separation membrane having a plurality of pores in at least a portion of the bottom surface thereof, which can retain cells that are dispersed thereon. Regarding the lower structure **13** as well, a lower structure comprising a bottom surface and an opening portion each having a round shape is given as an example. However, the shapes of the bottom surface and the opening portion are not limited to specific shapes. Further, it is not necessary that the bottom surface can be clearly distinguished from the lateral surface in the lower structure, and the lower structure may also be configured to contain a partial surface of a sphere in which the bottom surface is consecutive with the lateral surface.

[0125] The thus-described membrane separation culture device **1** can be used to separate stem cells from the tissues or cells of any given organisms including mammals. Examples of the stem cells that can be separated herein include embryonic stem cells, iPS cells, and tissue stem cells. The membrane separation culture device **1** can be used to separate dental pulp stem cells or mesenchymal stem cells from dental pulp cells or mesenchymal cells, in particular, for the purpose of regenerating the dental pulp of mammals including humans. Examples of mesenchymal stem cells include bone marrow stem cells, adipose stem cells, amniotic stem cells, and cord blood stem cells. However, the intended use of the membrane separation culture device **1** is not limited thereto.

[0126] Specifically, the above-described dental pulp stem cells or other tissue stem cells as targets of separation preferably comprise at least any one of CD105-positive cells, CXCR4-positive cells, SSEA-4-positive cells, FLK-1-positive cells, CD31-negative and CD146-negative cells, CD24-positive cells, CD150-positive cells, CD29-positive cells, CD34-positive cells, CD44-positive cells, CD73-positive cells, CD90-positive cells, FLK-1-positive cells, G-CSFR-positive cells, and SP cells, which are derived from the dental pulp or other tissues (e.g., bone marrow, adipose tissues, amnion, periodontal membrane, synovial membrane, or umbilical cord). The SP cells are preferably any one of CXCR4-positive cells, SSEA-4-positive cells, FLK-1-positive cells, CD31-negative and CD146-negative cells, CD24-positive cells, CD105-positive cells, CD150-positive cells, CD29-positive cells, CD34-positive cells, CD44-positive cells, CD73-positive cells, CD90-positive cells, FLK-1-positive cells, and G-CSFR-positive cells.

[0127] Next, the membrane separation culture device **1** will be described from the viewpoint of a method for separating stem cells. The method of separating stem cells comprises a step of dispersing test cells **200** or test tissues on a separation membrane **12** of an upper structure **10**, a step of pouring a medium **300** containing cell migration factor(s) into a lower structure **13**, and a step of allowing the separation membrane **12** to come into contact with the medium **300**. By these steps, stem cells are allowed to selectively pass from the upper portion of the separation membrane **12**, using the concentration gradient of the cell migration factor(s) placed in the lower structure **13** so that the stem cells can be separated.

[0128] In the step of dispersing the test cells **200** or test tissues on the separation membrane **12** of the upper structure **10**, the test cells **200**, which can be obtained by a known method, for example, according to Nakashima, Archs. Oral Biol. 36, 1991, are dissolved in a medium **100**, and the thus-obtained solution is then dispersed on the separation membrane **12** of the upper structure **10**. The test cells **200** used as a source for separation of stem cells may be dental pulp cells or mesenchymal cells. The mesenchymal cells include cells derived from bone marrow, adipose tissues, amnion, periodontal membrane, synovial membrane, or umbilical cord, but the examples of the mesenchymal cells are not limited thereto. In addition, when embryonic stem cells or iPS cells are separated, the test cells **200** used as a separation source can be an embryo, a blastocyst, or somatic cells on which gene introduction or protein introduction has been performed. When the test tissues are used, the tissues are minced, are immersed in a medium, and are then left at rest on the separation membrane **12** of the upper structure **10**.

[0129] The test cells are dispersed on the separation membrane at a cell density of 3×10^2 cells to 3×10^4 cells per mm^2 of the separation membrane. For example, the cell density is preferably 1×10^2 cells/100 μl to 1×10^7 cells/100 μl, and more preferably 1×10^4 cells/100 μl to 1×10^6 cells/100 μl, with respect to a separation membrane with a diameter of 6.5 mm. This is because if the cell density is too low, the cells hardly proliferate, and if the cell density is too high, the cells hardly migrate. The quantities of necessary test cells are different depending on the type of stem cells to be separated. For instance, only very small quantities (e.g., approximately 1×10^5 cells) of test cells that are dental pulp tissues are needed to separate 1×10^3 dental pulp stem cells. The most preferred density of test cells, in particular, in the case of separating dental pulp stem cells is 3×10^2 to 1.5×10^3 cells/mm^2. On the other hand, the densities of test cells required for separating the same quantities of bone marrow stem cells or adipose stem cells as dental pulp stem cells may be, for example,

approximately 3×10^5 cells and 1×10^6 cells, respectively. Moreover, when iPS cells are separated, the quantities of the test cells **200** are different depending on introduction efficiency. The necessary quantities of test cells are already known to those skilled in the art and, thus, can be determined as appropriate. Furthermore, when test tissues are used, the test tissues can be left at rest at a density of 0.1 mg to 1 mg per mm^2 of the separation membrane.

[0130] In the step of pouring the medium **300** containing cell migration factor(s) into the lower structure **13**, the cell migration factor(s) are dissolved in the medium, and the obtained solution is then poured into the lower structure **13**. The cell migration factor(s) added to a medium that is to be placed in the lower structure **13** are preferably at least any one of SDF-1, G-CSF, bFGF, TGF-β, NGF, PDGF, BDNF, GDNF, EGF, VEGF, SCF, MMP3, Slit, GM-CSF, LIF, HGF, S1P, protocatechuic acid, and serum. Particularly, from the viewpoint of migration activity and safety in clinical use, G-CSF or bFGF is most preferable. Moreover, the concentration of the cell migration factor(s) is preferably 1 ng/ml to 500 ng/ml. If the concentration is too low, there may be cases in which migration effect cannot be obtained. If the concentration is too high, there is a risk that differentiation of stem cells may occur. In particular, when G-CSF or bFGF is used as such a cell migration factor, the concentration of G-CSF or bFGF is preferably 50 to 150 ng/ml, and particularly preferably around approximately 100 ng/ml, for example, 95 to 105 ng/ml. This is because, using the cell migration factor in such a concentration, the largest quantities of stem cells can be separated and, further, the expression level of the mRNA of an angiogenic factor or a neurotrophic factor is high in the thus separated stem cells.

[0131] As a medium, Dulbecco's Modified Eagle Medium, EBM2, and the like can be used. However, the medium used herein is not limited thereto. Any medium, which can be used for the culture of stem cells, may be used. The amount of the medium can be determined, as appropriate, depending on the volume of the lower structure **13**. Serum, as well as cell at least one migration factor, is preferably added to the medium. This is because serum has the effect of promoting cell migration activity. To separate human stem cells, human serum is preferably used. Also, fetal bovine serum can be used. Such serum is preferably added in an additive amount of 5 to 20 vol % based on the volume of the medium.

[0132] In the step of allowing the separation membrane **12** to come into contact with the medium **300**, the upper structure **10** is laminated on the lower structure **13** so that the external side of the separation membrane **12** is allowed to sufficiently come into contact with the medium **300**. Thereby, the concentration gradient of the at least one cell migration factor can be carried out. As a result, stem cells contained in the test cells **200** or test tissues are allowed to pass through pores **121**, and they migrate towards the lower structure **13** so that the stem cells can be separated. The operation time required after the contact of the separation membrane with the medium is preferably 40 to 50 hours.

[0133] In the above explanation, the test cells **200** or the test tissues are placed in the upper structure **10**, and the medium containing cell migration factor(s) is placed in the lower structure **13** and, thereafter, the upper structure **10** is mounted on the lower structure so that the separation membrane **12** is allowed to come into contact with the medium. However, the order of performing these three steps is not determined. It may also be possible that the upper structure **10** is first combined with the lower structure **13**, and that dispersion and pouring are then carried out on the individual structures. Also, these operations may be carried out substantially simultaneously.

[0134] This method of culturing stem cells can be carried out regardless of the shape of a specific vessel. In such a case, the method of culturing stem cells comprises a step of allowing test cells or test tissues to come into contact with a non-cell-adhesive surface of a separation membrane having pores, and a step of allowing a medium containing cell migration factor(s) to come into contact with the other surface of the separation membrane.

[0135] Using the membrane separation culture device **1** and a method of separating stem cells using the same, stem cells can be separated safely and efficiently.

[0136] According to a second example, we provide a membrane separation culture device comprising a gas exchange system and a medium replacement system. FIG. **2** is a conceptual view showing a membrane separation culture device **2** according to this example. The membrane separation culture device **2** further comprises a lid structure **24**, as well as an upper structure **20** and a lower structure **23**.

[0137] The basic structures, materials and functions of the upper structure **20** and the lower structure **23** are the same as those described in the first example. In this example, the lower structure **23** further comprises a medium inlet port **231** and a medium outlet port **232**. These ports constitute the medium replacement system. The medium inlet port **231** and the medium outlet port **232** are opening portions that connect the inside of the lower structure **23** with the outside. With such opening portions, cell migration factor(s) and/or a medium containing the separated stem cells can be transferred between the lower structure **23** and the outside. That is, a medium d containing the separated stem cells can be removed through the medium outlet port **232**, and a fresh medium c containing cell migration factor(s) can be incorporated through the medium inlet port **231**. Therefore, the medium inlet port **231** and the medium outlet port **232** may be connected with a tube that is not shown in FIG. **2**. With such a medium replacement mechanism, the membrane separation culture device is advantageous in that a non-open system (hermetically sealed system) capable of complying with GMP avoids bacterial, viral and mycoplasma infection, and enhances safety and efficiency.

[0138] On the other hand, the lid structure **24** is a lid structure that is mounted on the upper structure **20** to cover the opening portion of the upper structure **20** and the opening portion of the lower structure **23**. It is preferable that the lid structure adhere tightly to the lower structure **23**, or to both the upper structure **20** and the lower structure **23** so that the membrane separation culture device **2** can be hermetically sealed from outside air. For the purpose of hermetically sealing the culture device, the lower structure **23** may be equipped with a rubber or silicone packing at the top edge of the lateral surface thereof.

[0139] The lid structure **24** further comprises a gas inlet port **241** and a gas discharge port **242**. These ports constitute a gas exchange system. The gas inlet port **241** and the gas discharge port **242** are opening portions that communicate the inside of the lid structure with the outside. The gas inlet port **241** and the gas discharge port **242** may be connected with a tube that is not shown in the Figures. For example, inert gas such as CO_2 or N_2 can be supplied into the membrane sepa-

ration culture device **2** via the gas inlet port **241**, and the used gas b such as CO_2 can be removed via the gas discharge port **242**.

[0140] The lid structure **24** may further comprise a silicone membrane having a plurality of pores having a pore size of 100 nm or less, particularly 1 to 100 nm, and preferably 10 to 100 nm. The silicone membrane can be configured to cover the gas inlet port **241** and the gas discharge port **242** in the lid structure. This is because invasion of mycoplasma from outside is blocked. With such a gas exchange system **24**, the membrane separation culture device is advantageous in that a non-open system (hermetically sealed system) capable of complying with GMP enhances safety, efficiency, and survival rate.

[0141] The membrane separation culture device may further comprise a temperature control system that is not shown in FIG. **2**. The temperature control system is composed of a temperature-measuring device for measuring the temperature inside the hermetically sealed membrane separation culture device **2** and a heater/cooler for heating or cooling the membrane separation culture device **2** from the outside thereof. With such a temperature control system, it becomes possible for the membrane separation culture device to manage the control of temperature, for example, using a temperature-sensitive medium system for the lower structure **23**.

[0142] The membrane separation culture device **2** comprising both a medium replacement mechanism and the gas exchange system **24** is described. However, the membrane separation culture device is not limited thereto, and it may comprise either one of them. Using the membrane separation culture device **2** according to the second example, a culture that does not particularly need medium replacement can be carried out more safely by a circulating system, and thus, stem cells preferable for tissue regeneration can be efficiently obtained.

[0143] According to a third example, we provide a membrane separation culture device comprising a lid structure. FIG. **3** is a conceptual view showing the configuration of a membrane separation culture device **3** according to this example. The membrane separation culture device **3** further comprises a lid structure **34**, as well as an upper structure **30** and a lower structure **33**.

[0144] The upper structure **30** is a vessel composed of an axial portion having a round-shaped bottom surface and a circular truncated cone portion having an opening portion. The upper structure **30** is configured such that the diameter of the opening portion is greater than the diameter of the bottom surface. The opening portion of the circular truncated cone portion comprises a retention mechanism **35** consisting of a flange that extends outside.

[0145] The lower structure **33** shown in FIG. **3** is a cylindrical member comprising a round-shaped bottom surface and a round-shaped opening portion, wherein they have the same diameter. The lower structure comprises a groove close to the opening portion, and retains a hermetic sealing elastic body **331** in the groove. The groove may be either a single groove or a double groove. The material of the hermetic sealing elastic body used herein is desirably a synthetic rubber having a clear composition such as a silicone rubber. Moreover, around the center of the cylinder and between the groove in which the elastic body **331** is established and the bottom surface, the lower structure **33** also comprises a retention mechanism **335** consisting of a flange extending from the inner wall surface of the cylinder towards the inside. The retention mechanism **335** engages with the retention mechanism **35** of the upper structure **30** to retain the upper structure **30** at a predetermined position in the lower structure **33**.

[0146] The basic structures, materials and functions of the upper structure **30** and the lower structure **33**, other than the above descriptions, are the same as those described in the first example.

[0147] The lid structure **34** is a member capable of covering or hermetically sealing the upper structure **30** and the lower the structure **33**. The lid structure **34** may cover the opening portions of the upper structure **30** and the lower structure **33** from the above of the upper structure **30**. The material of the lid structure is the same as that of the lower structure described in the first example. It is desirable that the lid structure **34** further comprise a gas exchange mechanism (not shown in FIG. **3**). This gas exchange mechanism may be a plurality of pores having a pore size of 100 nm or less, preferably 1 to 100 nm, and particularly preferably 10 to 100 nm, which are established on the entire surface or a portion of the lid structure **34**. As another example of the gas exchange mechanism, a polymer membrane for passing gas such as a polytetrafluoride ethylene (PTFE) laminated membrane used for gas line filters, may be established in at least a portion of the lid structure **34**. The lid structure **34** may further comprise a gas inlet port and a gas discharge port, as described in the second example.

[0148] The lid structure **34** is combined with the lower structure **33** so that it can be tightly adhered to the elastic body **331** retained in the lower structure **33**. By such a configuration, hermetic sealing can be carried out simply. This membrane separation culture device further comprises a gas exchange function (not shown in FIG. **3**) and, as a result, it provides a structure that gives no pressure change to stem cells.

[0149] With such a lid structure **34** and a lower structure **33**, the membrane separation culture device according to this example is advantageous in that a risk of contamination can be reduced in the case of covering the lower structure with the lid structure, and in that contamination such as mycoplasma can be avoided and a pressure change due to a temperature change can also be avoided in the case of hermetically sealing the lower structure with the lid structure.

[0150] According to a fourth example, we provide a membrane separation culture device comprising a plurality of upper structures. FIG. **4** is a conceptual view showing the configuration of a membrane separation culture device **4** according to this example. The membrane separation culture device **4** comprises a plurality of upper structures **40**, a frame body **45** for holding the plurality of the upper structures **40**, and a lower structure **43** constituted with a vessel for collectively retaining a fluid in which the separation membranes of the plurality of the upper structures **40** are immersed.

[0151] The structure of each upper structure **40** may be the same as that in the third example. The plurality of the upper structures **40** may comprise separation membranes each having a different pore size and/or a different pore density, or all of the separation membranes may have the same pore size and/or the same pore density.

[0152] The frame body **45** is a structure that is to be contained in the lower structure **43**, and it contains the plurality of the upper structures **40** in individual holes **451**. Specifically, the frame body **45** is a member in which upper and lower parts are opened, wherein the frame body **45** comprises a plurality of holes **451** established on a plate-like member that is

retained at a constant height by a retention mechanism **453**. A grid-like partition **452** divides the upper region of the plate-like member to form partitions, and a single hole **451** is present in one partition. The material of the frame body **45** may be the same as that of the lower structure described in the first example. In addition, the retention mechanism **453** is a pedate member extending from the periphery of the plate-like member to the lower portion. The retention mechanism **453** is formed like an outer frame only around the periphery of the plate-like member, and a slit is formed on a portion corresponding to the partition **452** on the upper surface of the plate-like member. FIG. **4** shows the structure of the frame body **45** having 24 holes. However, the number of holes formed on the frame body **45** may be either 2 or 96, and the number of holes is not limited. Moreover, the disposition of holes established on the plate-like member of the frame body **24** is not limited to the example shown in the figure. Furthermore, the retention mechanism **453** in this example is a pedate member extending from the plate-like member downwards. A flange extending from the inner wall of the lower structure to the inside may be established so that the retention mechanism **453** may be moored at the flange.

[0153] The upper structure **40** can be detachably inserted into each hole **451** on the frame body **45**. The hole **451** holds the upper structure **40** such that when the upper structure **40** is inserted, the separation membrane constituting the bottom surface of the upper structure **40** can be positioned between the hole **451** and the inner wall of the bottom surface of the lower structure **43**. That is, the hole **451** is formed such that the diameter of the hole **451** it becomes greater than the diameter of the bottom surface of the upper structure **40** and also becomes smaller than the diameter of the opening portion.

[0154] The lower structure **43** is a vessel for detachably containing the frame body **45**. A single groove is established on the lower structure **43**. In the groove, the elastic body **431** described in the third example is established. The elastic body **431** is configured to be hermetically attached to the aforementioned lid structure **44** to hermetically close the inside of the membrane separation culture device **4**. Other structures, materials, and functions of the lower structure **43** are the same as those described in the first example. The lower structure **43** may further comprise a medium inlet port and a medium outlet port (not shown in FIG. **4**). The lower structure shown in FIG. **4** has a square bottom surface. However, the shape of the lower structure is not limited to a square shape, and it may be circular or elliptical, for example.

[0155] The lid structure **44** covers an opening portion between the plurality of the upper structures **40** and the lower structure **43** from the above of the plurality of the upper structures **40**. The basic structure, material, and function of the lid structure **44** are the same as those described in the third example. The lid structure **44** may comprise a gas exchange mechanism (not shown in FIG. **4**) as described in the third example, or may further comprise a gas inlet port and a gas discharge port, as well as the gas exchange mechanism.

[0156] By comprising such a frame body **42** and a lower structure **43**, the membrane separation culture device is advantageous in that large quantities of tissues or cells can be used as analytes and they can be collected by a single lower structure, when the number of stem cells of interest is small, as in the case of iPS cells.

[0157] According to a fifth example, we provide a membrane separation culture device comprising a plurality of upper structures and a lower structure composed of a plurality of vessels. FIG. **5** is a conceptual view showing the configuration of a membrane separation culture device **5**.

[0158] The membrane separation culture device **5** comprises a plurality of upper structures **50**, a frame body **55**, and a lower structure **53** composed of a plurality of vessels each retaining a fluid in which a separation membrane of each upper structure **50** is immersed.

[0159] The basic structure and function of the upper structure **50** are the same as those described in the fourth example. In this example as well, the plurality of the upper structures **50** may comprise separation membranes each having a different pore size and/or a different pore density, or all of the separation membranes may have the same pore size and/or the same pore density.

[0160] The basic structure and function of the frame body **55** are the same as those described in the fourth example. In this example, a retention mechanism **553** as a lower portion of the frame body **55** is equipped with a plurality of slits to avoid interference with partitions **532** of the lower structure **53**.

[0161] On the other hand, the lower structure **53** is composed of a plurality of vessels each corresponding to the plurality of the upper structures. Specifically, the main body of the lower structure consists of a plurality of vessels composed of divisions formed by dividing with grid-like partitions **532**. The grid-like partitions **532** may be formed by being integrated with the lower structure **53**, or may be a member detachable from the lower structure **53**. The partitions **532** are fixed on the lower structure to such an extent that substances cannot move between individual vessels formed with such partitions **532** upon use.

[0162] The frame body **55** can be mounted on the lower structure **53**, and thus, the lower structure **53** can contain the frame body **55**. When the frame body **55** can be mounted on the lower structure **53**, individual holes **551** of the frame body **52** each correspond to a plurality of vessels of the lower structure **53**. In addition, partitions **552** of the frame body **55** are overlapped with the partitions **532** of the lower structure **53**. Moreover, each of the plurality of upper structures **50** can be mounted on each hole **551** of the frame body **52**. At this time, a combination of one vessel of the lower structure **53** with one upper structure **50** functions as an independent membrane separation culture device. Therefore, each of the vessels divided with the partitions **532** can retain a fluid in which the separation membrane of the upper structure **50** is immersed. For example, different types of fluids containing different migration factor and/or media are placed in different vessels to carry out membrane separation.

[0163] The membrane separation culture device **5** may also comprise a lid structure that is not shown in FIG. **5**. The lid structure may comprise the gas exchange mechanism (not shown in FIG. **5**) described in the third example, or may further comprise a gas inlet port and a gas discharge port, as well as the gas exchange mechanism. The lower structure **53** shown in FIG. **5** does not have a groove used for hermetically sealing, and it can be used in combination with a covering lid structure.

[0164] Using the membrane separation culture device **5** according to the fifth example to separate stem cells that have not yet been separated so far, a plurality of upper structures having different pore sizes are prepared, and a plurality of media containing various cell migration factor(s) are prepared and combined with the upper structures so that separation conditions can be advantageously screened at one time.

[0165] According to a sixth example, we provide a closed system membrane separation culture device capable of carrying out subculture. FIG. **22** is a conceptual view showing the configuration of a membrane separation culture device **6** according to this example.

[0166] The membrane separation culture device **6** is essentially composed of a dish **66**, as well as an upper structure **60** and a lower structure **63**. The basic structure, material, and function of the upper structure **60** are the same as those described in the first example. A lid structure **64** for covering the opening portion of the upper structure **60** from the above of the upper structure **60** is established at the opening portion of the upper structure **60**. The lid structure **64** comprises an introduction port **65**. The introduction port **65** is a tube preferably made of silicone, which is established by penetrating through the lid structure **64**, and is used to communicate the inside of a vessel constituted with the upper structure **60** with the outside. The introduction port **65** is mainly used to insert minced dental pulp tissues or dental pulp test cells into the membrane separation culture device **6** in a closed system, without contamination of the tissues or cells from the outside. Using the introduction port **65**, it is also possible to replace a medium with another one in a closed system. The structure, material, and function of a membrane **62** comprised in the upper structure **60** are the same as those described in the first example. On the other hand, the lower structure **63** according to this example does not have a bottom surface portion integrated and fixed with a lateral surface portion thereof and is composed only of the lateral surface portion. Other than this, the lower structure **63** has the same configuration as that in the first example.

[0167] The dish **66** is a vessel composed of a bottom surface portion and a lateral surface portion, and is capable of cell culture. A recovery port **661** for recovering a medium is established on the dish **66**, and thereby, medium replacement, the recovery of a culture supernatant, and the recovery of cells can be carried out in a closed system without contamination from the outside. A surface treatment layer that is not shown in FIG. **22** is established on the bottom surface portion of the dish **66**, which is the inner surface of the vessel. It is preferable that the surface treatment layer has properties excellent in cell adhesion and amplification, be reacted by heat or light, or by both of them, and be degradable. According to one example, the surface treatment layer can be designed such that it is reacted by irradiation of light with a specific wavelength, and that as a result, a substance constituting the surface treatment layer is decomposed. As an example, poly(N-isopropylacrylamide) is graft polymerized onto the bottom surface portion of the dish **66**, which is the inner surface of the vessel to form a surface treatment layer. This surface treatment layer retains hydrophobicity at 37° C., but when the temperature is decreased to approximately 30° C., it is subjected to phase change and is thereby hydrophilized. Thus, it becomes possible to remove cells adhered to the surface of the layer. According to another example, the surface treatment layer can be designed such that it is reacted by a specific temperature change, and that as a result, a substance constituting the surface treatment layer is decomposed. As an example, a surface treatment layer comprising collagen can be formed. In this case, the surface treatment layer is decomposed by increasing the temperature to a collagen denaturation temperature, and as a result, it becomes possible to remove cells adhering to the surface of the layer.

[0168] The dish **66** further comprises, at the upper portion thereof, a lid structure **67** for covering the opening portion of the dish **66** from the above. The lid structure **67** is configured to hermetically seal the inside of the dish **66**, and to maintain a hermetically sealed state even if a laminated body of the upper structure **60** and the lower structure **63** moves in the vertical direction.

[0169] In this example, the lower structure **63** is established movably in the vertical direction in the dish **66** and is used. FIG. **22**(*a*) is a conceptual view showing the disposition of individual components when membrane separation is carried out. At this time, the membrane **62**, the lateral surface portion of the lower structure **63** and the bottom surface portion of the dish **66** form a closed space, and cells migrating from the upper structure **60** are retained in the space. The diameter of the dish **66** is preferably about 7 to 10 times larger than the diameter of the lower structure **63**. It is to be noted that the scale used in FIG. **6** is changed to clearly display each member.

[0170] It is possible that the lower structure **63** be moved upward in the vertical direction and is then fixed. FIG. **22**(*b*) is a conceptual view showing the membrane separation culture device **6** when the lower structure **63** is moved upward in the vertical direction for medium replacement or the recovery of stem cells. In this case as well, the lid structure **67** can hermetically seal the dish **66**.

[0171] Next, the membrane separation culture device **6** according to the sixth example will be described from the viewpoint of a closed system culture method. Such a closed system culture method comprises a step of carrying out membrane separation, a step of allowing stem cells to proliferate, a step of subculturing the step cells, and a step of amplifying the step cells and recovering them. In the step of carrying out membrane separation, stem cells are separated by a membrane according to the method described in the first example. In this step, cells that have migrated from the upper structure **60** to the lower structure **63** adhere to the bottom surface of the dish **66** enclosed with the side wall portion of the lower structure **63**. Subsequently, in the step of allowing stem cells to proliferate, a laminated body of the upper structure **60** and the lower structure is moved upward in the vertical direction, and the medium is then replaced with a cell growth medium such as DMEM containing 10% serum through the recovery port **661** to remove migration factors, and the laminated body of the upper structure **60** and the lower structure is then moved downward in the vertical direction so that it is allowed to come into contact with the bottom surface portion of the dish **66** and is fixed thereon, thereby allowing stem cells to proliferate. Thereafter, the step of subculturing the stem cells is carried out. During this step, the laminated body of the upper structure **60** and the lower structure is moved upward in the vertical direction and is then fixed. Then, light or heat is applied onto the surface treatment layer, or the temperature is decreased so that the surface layer is decomposed and the proliferating stem cells are removed therefrom. At this time, since the side wall portion of the lower structure **63** is not allowed to come into contact with the bottom surface portion of the dish **66**, the stem cells are diffused over the entire dish **66**. In the step of amplifying the stem cells and recovering them, the subcultured stem cells can be recovered through the medium recovery port **661**. During this step, a centrifuge tube is connected with the recovery port **661**, and centrifugation is then performed in a closed system to recover cells or a cell

supernatant. Moreover, in each step, medium replacement can be carried out through the medium recovery port **661**.

[0172] In this example, by establishing a surface treatment layer on the dish **66**, it becomes unnecessary for the removal of cells to use an enzyme that has conventionally been used to remove cells from a culture vessel such as trypsin. Since the obtained cell culture supernatant and cells do not contain enzyme, they can be directly used in transplantation without centrifugation and washing.

[0173] According to a seventh example, we provide a membrane separation culture kit. The membrane separation culture kit comprises a membrane separation culture device and cell migration factor(s). As a membrane separation culture device, any of the membrane separation culture devices 1 to 6 according to the above described first to sixth examples, or modified forms thereof can be used.

[0174] The cell migration factor is preferably at least one of SDF-1, G-CSF, bFGF, TGF-β, NGF, PDGF, BDNF, GDNF, EGF, VEGF, SCF, MMP3, Slit, GM-CSF, LIF, HGF, S1P, protocatechuic acid, and serum. In addition, the concentration of the cell migration factor is preferably 1 ng/ml to 500 ng/ml. This is for efficient migration of stem cells. That is to say, if the concentration of the migration factor is too low, the migration effect may not be obtained. In contrast, if the concentration is too high, the cells may be differentiated. The cell migration factor may be added to a medium and may be then used. Accordingly, the kit according to this example may also comprise a medium. As a medium, Dulbecco's modified Eagle's medium, EBM2, or the like can be used, but examples of the medium used herein are not limited thereto.

[0175] Particularly preferably, the kit comprises, as a kit-constituting member, a cell migration factor that is G-CSF or bFGF, preferably in a concentration of 50 to 150 ng/ml. Moreover, in addition to the cell migration factor, the kit may also comprise, as a kit-constituting member, another component to be added to a medium that is human autoserum or fetal bovine serum. Such a serum is configured to be added in a concentration of 5 vol % to 20 vol % based on the total volume of the medium. The kit can be produced and used in accordance with the explanations regarding the devices and methods of the first to fifth examples.

[0176] According to the membrane separation culture kit of this example, using a membrane separation culture device and cell migration factor(s) used for separation, the method of separating stem cells that is specifically described in the first example can be promptly carried out.

[0177] According to an eighth example, we provide a separation membrane.

[0178] The separation membrane according to this example comprises: a base material membrane consisting of a hydrophobic polymer; and a functional layer formed by allowing one or more hydrophilic polymers selected from a vinyl pyrrolidone polymer, a polyethylene glycol polymer and a vinyl alcohol polymer to bind to the surface of the base material membrane via a covalent bond, wherein the weight percentage of the hydrophilic polymer(s) constituting the functional layer is 1.5% to 35% based on the total weight of the separation membrane.

[0179] Since the remaining organic solvent may have adverse effects on cells such that the pores of the separation membrane may be clogged with the remaining organic solvent, or elution may occur with an aqueous culture medium, it is preferable that membrane surface modification be carried out on the separation membrane according to a covalent bond method. Specifically, a base material membrane, on which pores each having a desired pore diameter have been formed at a high porosity rate, is immersed in a treating aqueous solution, to which a vinyl pyrrolidone polymer, and/or ethylene glycol polymer, and/or vinyl alcohol polymer, and optionally, an alcohol have been added. Thereafter, the base material membrane is irradiated with a high-energy beam to the surface of the base material membrane can be modified.

[0180] The polymer used as a base material membrane of the separation membrane is preferably a hydrophobic polymer. The term "hydrophobic polymer" indicates a polymer whose solubility in 100 g of water at 20° C. is less than 0.001 g. The hydrophobic polymer is specifically selected from the group consisting of a sulfone polymer an amide polymer, a carbonate polymer, an ester polymer, a urethane polymer, an olefin polymer, and an imide polymer, but examples of the hydrophobic polymer are not limited thereto. Surface modification conditions are changed based on the water absorption percentage of such a hydrophobic polymer constituting the base material membrane so that a protein or cell adhesion-suppressing property can be more efficiently imparted to the separation membrane.

[0181] The base material membrane does not need to have pores, as long as it is a membrane for separating two regions. For permeation of a substance, a membrane having pores with a diameter of 40 to 80 nm such as a dialytic membrane, may be used, and for cell separation, a membrane having pores with a diameter of 1 to 10 μm may be used. Thus, a pore diameter may be selected depending on intended use. In particular, the separation membrane can be preferably used for separation in which cell chemotaxis is utilized. In this case, a pore diameter of 3 to 8 μm is easily used.

[0182] The polymer constituting such a base material membrane generally has strong hydrophobicity, and it is likely that many proteins or cells adhere thereto. In particular, since activated proteins or platelets, or adherent cells easily adhere onto the surface of the membrane, it has been concluded that a certain level of surface modification needs to be uniformly carried out, namely, that the covalent bond of the hydrophobic polymer with a hydrophilic polymer is necessary.

[0183] The vinyl pyrrolidone polymer is a polymer selected from the group consisting of polyvinyl pyrrolidone, a vinyl pyrrolidone/vinyl acetate copolymer, a vinyl pyrrolidone/vinyl alcohol copolymer, a vinyl pyrrolidone/styrene copolymer, a vinyl pyrrolidone/dimethyl aminoethyl methacrylate copolymer, and a modified polymer thereof. The ethylene glycol polymer includes those containing an ester group on the side chain thereof. As vinyl alcohol polymers, various types of polymers can be obtained depending on saponification degree. However, the type of such a vinyl alcohol polymer is not limited. These polymers are referred to as hydrophilic polymers. These hydrophilic polymers used for membrane surface modification are preferably water-soluble. Thus, polymers having a number average molecular weight of 10,000 to 1,000,000 can be used, for example. However, as long as the polymer is water-soluble, its molecular weight is not limited to the aforementioned molecular weight.

[0184] The term "water-soluble polymer" means a polymer for which solubility in 100 g of water at 20° C. is 1 g or more, and preferably 10 g or more. From the viewpoint of suppression of adhesion of proteins, platelets, adherent cells and the like, the separation membrane preferably contains such a water-soluble polymer. An appropriate balance between hydrophilicity and hydrophobicity on the surface has been

considered important for suppression of adhesion of proteins or platelets. As a matter of fact, it was found that, when a water-soluble polymer having stronger hydrophilicity is present, the effect of suppressing the adhesion of proteins, platelets, adherent cells and the like is further improved.

[0185] The amount of a water-soluble polymer contained in the separation membrane is preferably 1.5% or more, and more preferably 5% or more, based on the total weight of the separation membrane. In addition, since the effect is not changed even if the separation membrane contains an excessively large amount of water-soluble polymer, the upper limit of the water-soluble polymer is preferably 40% or less, and more preferably 35% or less, based on the total weight of the separation membrane.

[0186] Furthermore, if the hydrophilic polymer is a copolymer having a water-soluble unit and an ester group unit, it has an appropriate balance between hydrophilicity and hydrophobicity in a single molecule thereof. Thus, the hydrophilic polymer is preferably such a copolymer. As such a copolymer, a block copolymer, an alternating copolymer, and a random copolymer are preferably used, rather than a graft copolymer. This is because, in the case of a graft copolymer, since a unit portion grafted to a main chain is allowed to often come into contact with a protein or the like, the properties of a graft chain portion become greater than the properties of a copolymer. Further, an alternating copolymer and a random copolymer are more preferable than a block copolymer. This is because, in the case of a block copolymer, the properties of individual units are clearly different from one another. In terms of a balance between hydrophilicity and hydrophobicity in a single molecule, a copolymer having at least one selected from a random copolymer and an alternating copolymer is preferably used. The molar ratio of an ester group unit in an ester group-containing polymer is preferably 0.3 or more and 0.7 or less. If the molar ratio of the ester group unit is less than 0.3, the adhesion-suppressing effect of the ester group is reduced. On the other hand, if the molar ratio of the ester group unit exceeds 0.7, the effect of the water-soluble unit is reduced.

[0187] The amount of a hydrophilic polymer serving as a surface-modifying polymer on the surface of a separation membrane can be measured, for example, by elementary analysis, nuclear magnetic resonance (NMR) measurement, or a combination of ESCA and attenuated total reflection method (hereinafter also referred to as ATR). This is because ESCA is used to measure a depth of about 10 nm from the surface, whereas ATR is used for surface measurement that is the measurement of the composition of a depth of several μm. Taking a polysulfone separation membrane as an example, when the ratio of the amount of a hydrophilic polymer to the amount of a polysulfone unit in any given site in the membrane is defined as a unit amount ratio, if the value of the unit amount ratio obtained by ESCA is 30% or more greater than the value obtained by ATR, it can be determined that the amount of an ester group-containing polymer on the membrane surface is 30% or more greater than the amount inside the membrane. It is to be noted that the value of each measurement is indicated as a mean value of three sites.

[0188] Next, this example will be described from the viewpoint of a method of modifying the surface of a molded body. The method of modifying the surface of a molded body comprises: an immersion step of immersing a molded body consisting of a hydrophobic polymer in a treating aqueous solution containing one or more hydrophilic polymers selected from a vinyl pyrrolidone polymer, a polyethylene glycol polymer and a vinyl alcohol polymer at a concentration of 10 to 2000 ppm, and further optionally containing a 0.01 wt % to 0.2 wt % alcohol; and a modification step of irradiating the molded body obtained by the immersion step with a high-energy beam to modify the surface of the molded body to have a protein adhesion-suppressing property and a cell adhesion-suppressing property. When the molded body is a specific base material membrane, such a method of modifying the surface of a molded body can also be referred to as a method of producing a separation membrane, as described above. This method is preferable because it can be easily carried out with a small amount of treating solution.

[0189] The molded body consisting of a hydrophobic polymer as defined herein is not limited to a membrane, and it may also be a molded body having a specific shape. When a membrane is used as such a molded body, it may be a base material membrane described in the configuration of the above described separation membrane. In such a case, the method of modifying the surface of a molded body may be equal to a method of producing a separation membrane.

[0190] The treating aqueous solution is an aqueous solution, in which a molded body, consisting of a hydrophobic polymer is to be immersed. The treating aqueous solution contains one or more hydrophilic polymers selected from a vinyl pyrrolidone polymer, a polyethylene glycol polymer and a vinyl alcohol polymer, at a total concentration of preferably 10 to 5,000 ppm, and more preferably 10 to 2,000 ppm. It is to be noted that a specific concentration may be different depending on the type of a hydrophilic polymer. If the concentration of the hydrophilic polymer solution is low, there is a case in which a molded body consisting of a hydrophobic polymer may not be sufficiently coated with the solution. On the other hand, if the concentration is too high, when the molded body is a membrane, for example, pores may be clogged, the amount of an elution product may be increased, or the performance of a separation membrane may be reduced in many cases. As described later, surface modification can be more efficiently carried out by addition of an alcohol. In such a case, the aforementioned concentration can be set lower than that as mentioned herein.

[0191] Moreover, to efficiently carry out surface modification, it is preferable to change the composition of the treating aqueous solution depending on the water absorption percentage of the material for the molded body consisting of a hydrophobic polymer. The water absorption percentage of the molded body consisting of a hydrophobic polymer can be obtained by washing with purified water, a membrane having a thickness of 30 to 100 μm that is a material for the molded body, then drying the membrane, and then immersing the dried membrane in water at 23° C. for 24 hours, followed by measuring an increased percentage in weight. This weight increase percentage is defined as a water absorption percentage. On the other hand, when the molded body is a separation membrane having a thickness of 200 μm or less, it is directly washed with purified water, is then dried, and is then immersed in water at 23° C. for 24 hours, and thereafter, the water absorption percentage can be calculated from a weight increase percentage during this operation.

[0192] When the water absorption percentage of a molded body consisting of a hydrophobic polymer is 2% or less, it is preferable to further add an alcohol to a treating aqueous solution used. This is because the surface of the molded body consisting of a hydrophobic polymer can be uniformly coated

with the treating aqueous solution as a result of the coexistence of the alcohol. Taking into consideration safety in a case in which the treating aqueous solution remains, the alcohol added is preferably ethanol, but examples of the alcohol added are not limited thereto. It is also possible to use a polyhydric alcohol such as glycerin. The concentration of the alcohol added is preferably 1% or less based on the total weight of the treating aqueous solution. For the sake of safety, it is more preferably 0.5% or less, and further preferably 0.1% or less, based on the total weight of the treating aqueous solution. Since surface modification can be efficiently carried out by previously enhancing an adsorption percentage with an alcohol, the same level of surface modification can be realized even with the use of a lower concentration of hydrophilic polymer. That is to say, the amount of a hydrophilic polymer used can be reduced, and it is effective for cost reduction during production.

[0193] On the other hand, when the water absorption percentage of a molded body consisting of a hydrophobic polymer exceeds 2%, it is not necessary to add an alcohol to a treating aqueous solution.

[0194] In the immersion step, the entire molded body may be immersed in the treating aqueous solution, or only a portion of the molded body that is to be subjected to surface modification may be immersed in the treating aqueous solution.

[0195] The high-energy beam used in the modification step may be UV, an electron beam, a γ-ray, or an X-ray. Of these, an electron beam or a γ-ray is more preferable because it easily enhances a reaction rate. In addition, in terms of a small amount of residual toxicity or simplicity, a γ-ray or an electron beam is preferably used. The applied dose is preferably 5 to 35 kGy. In particular, by irradiating the culture device as a whole with, for example, a dose of approximately 25 kGy that is considered to be a sterilization dose, surface modification and sterilization can be simultaneously carried out. However, if the applied dose is 100 kGy or more, productivity is reduced, and decomposition of a polymer and the like occurs. Thus, application of an excessively high dose is not preferable.

[0196] It has been known that, when the surface of the membrane is irradiated with a high-energy beam, if oxygen is present, oxygen radical or the like is generated, and a molded body consisting of a hydrophobic polymer is thereby decomposed. Accordingly, the oxygen concentration around the molded body is desirably 10 vol % or less during irradiation.

[0197] Since the separation membrane according to the eighth example has a high adhesion-suppressing property, it can be preferably used as a separation membrane for water treatment or a separation membrane for biological components. Moreover, the modification method according to this example can be applied, not only to membranes, but also to various types of molded bodies, and it can easily carry out surface modification at a high efficiency. This surface modification method is particularly suitable for a blood purification module. The blood purification module herein means a module having a function to circulate blood to the outside of the body and to remove waste products or harmful substances from the blood. Examples of such a module include an artificial kidney and an exotoxin adsorption column.

[0198] Hereinafter, our devices, kits, membranes and methods will be described more specifically in the following examples. However, these examples are not intended to limit the scope of this disclosure.

Example 1

Comparison of Migration Factors Effective for Migration of Dental Pulp Stem Cells by TaxiScan

[0199] For real-time horizontal chemotaxis analysis, the 4th-generation dog dental pulp stem cells $CD31^{-}SP$ were used. Using TAXIScan-FL (Effector Cell Institute, Tokyo), a channel optimized to the size of cells (8 μm) was formed between silicone having pores with a pore size of 6 μm and a glass plate, and 1 μl of cells (10^5 cells/ml) was then poured in one side of the channel. Various types of migration factors (10 ng/μl) were each poured into the opposite side thereof to form a certain concentration gradient. Based on video images of migration, the number of migrating cells was counted every 30 minutes until 4 hours after initiation of the operation. FIG. **6**(*a*) shows a difference in migration ability depending on the types of the migration factors over time. In the case of BDNF, the dental pulp stem cells migrated very promptly, and 2 hours later, the migration level reached plateau. In the case of SDF-1 and bFGF as well, migration progressed relatively promptly. In the case of GDNF, VEGF, MMP3, and G-CSF, the number of the migrating cells was gradually increased, and 4 hours later, the number of migrating cells became almost the same, except for PDGF and GM-CSF.

Concentration of Migration Factor Effective for Migration of Dental Pulp Stem Cells, Analyzed by TaxiScan

[0200] 1 μl of the dog dental pulp stem cells, $CD31^{-}SP$ (10^5 cells/ml) at the 4th passage of culture was poured in a TAXIScan-FL microchannel, and 1 μl each of G-CSF was then poured in the opposite side in a concentration of 0, 0.1, 1, 5, 10, 20, 40, or 100 ng/μl. Based on video images of migration, the number of migrating cells was counted every 30 minutes until 1.5 hours after initiation of the operation. FIG. **6**(*b*) shows a difference in migration ability depending on the concentration of G-CSF over time. In the case of 10 ng/μl G-CSF, the number of migrating cells is largest, and then, in the order of the concentration of 5, 40, 20, 100, 1, and 0.1 ng/μl, the number of migrating cells was decreased.

Concentration of Serum Effective for Migration of Dental Pulp Stem Cells, Analyzed by TaxiScan

[0201] 1 μl of human dental pulp stem cells (10^5 cells/ml) was poured in a TAXIScan-FL microchannel, and human serum was then poured in the opposite side in a concentration of 0%, 5%, 10%, 15%, or 20%. Based on video images of migration, the number of migrating cells was counted every 3 hours until 24 hours after initiation of the operation. Thereafter, a comparison was made on migration ability, in the case of using 10% and 20% human serums and 100 ng/ml G-CSF and SDF-1. FIG. **7**(*a*) shows a difference in migration ability depending on the concentration of serum over time. The number of migrating cells was largest in the case of 20% human serum, and then, in the order of the concentration of 15%, 10%, and 5%, the number of migrating cells was decreased. FIG. **7**(*b*) shows migration ability in the case of G-CSF or SDF-1 over time. The number of migrating cells in the case of G-CSF or SDF-1 was greater than the number of migrating cells in the case of 20% human serum.

Separation of Dental Pulp Stem Cells

[0202] There was assembled a membrane separation culture device composed of: a PET-made upper structure having

a bottom surface with a diameter of 6.4 mm, an opening portion with a diameter of 11.0 mm, and a height of 17.5 mm, wherein a non-cell-adhesively coated PET membrane (2×10^5 pores/cm^2, pore size: 3 μm) of Cell culture Insert was equipped into the bottom surface thereof; and a polystyrene-made lower structure having a bottom surface with a diameter of 15.0 mm, an opening portion with a diameter of 15.0 mm, and a height of 22.0 mm. To impart a cell adhesion-suppressing property to the surface of the PET-membrane, the PET membrane had previously been immersed in an aqueous solution prepared by adding 0.1% ethanol to a 1,000 ppm aqueous solution of a polyvinyl pyrrolidone-polyvinyl acetate copolymer (vinyl pyrrolidone/vinyl acetate (6/4) copolymer ("Kollidon VA64," manufactured by BASF)) to seal it. Thereafter, the membrane was modified by irradiation with a γ-ray (25 kGy), thereby preparing a separation membrane. On this membrane of the upper structure, fresh dog primary dental pulp cells were dispersed at a cell density of 1×10^5 cells/250 μl. On the other hand, G-CSF or SDF-1 was added into Dulbecco's modified Eagle's medium (DMEM) containing 10% dog serum in the lower structure, resulting in a final concentration of 100 ng/ml. Six hours later, the medium was replaced with fresh medium, and the G-CSF was then removed. The resultant was further cultured in DMEM containing 10% dog serum. FIGS. **8**(*a*), (*b*) and(*c*) each show a phase contrast microscopic image of dental pulp stem cells, which have migrated, adhered, and further proliferated. It became clear that, in all of the migration factors, star-like cells having projections adhered and proliferated, as in the case of separating $CD31^-$SP cells, $CD105^+$ cells or $CXCR4^+$ cells by flow cytometry.

Characterization of Separated Dental Pulp Stem Cells

[0203] The above described dental pulp stem cells, which had been membrane-separated using G-CSF and SDF-1 in the membrane separation culture device, were subcultured for three generations. Thereafter, the cells were dispersed in DMEM containing 2% serum at a cell density of 1×10^6 cells/ml, and were then labeled with various types of stem cell surface antigen marker antibodies (CD29, CD31, CD34, CD44, CD73, CD90, CD105, CD146, CD150, and CXCR4) at 4° C. for 30 minutes. Thereafter, flow cytometry was carried out. That is to say, the cells were labeled at 4° C. for 90 minutes using mouse IgG1 negative control (AbD Serotec Ltd.), mouse IgG1 negative control (fluorescein isothiocyanate, FITC) (MCA928F) (AbD Serotec), mouse IgG1 negative control (Phycoerythrin-Cy5, PE-Cy5) (MCA928C) (AbD Serotec), mouse IgG1 negative control (Alexa 647) (MRC OX-34) (AbD Serotec), antibodies to the following: CD29 (PE-Cy5) (MEM-101A) (eBioscience), CD31 (FITC) (Qbend10) (Dako), CD34 (Allophycocyanin,APC) (1H6) (R&D Systems, Inc.), CD44 (Phycoerythrin-Cy7,PE-Cy7) (IM7) (eBioscience), CD73 (APC) (AD2) (BioLegend), CD90 (FITC) (YKIX337.217) (AbD Serotec), anti-human CD105 (PE) (43A3) (BioLegend)CD146 (FITC) (sc-18837) (Santa Cruz,Biotech,Santa Cruz, Calif.,USA), CD150 (FITC) (A12) (AbD Serotec), CXCR4 (FITC) (12G5) (R&D). As a control, dog dental pulp $CD105_+$ cells, which had been separated by flow cytometry, were used.

[0204] Table 1 shows the expression of surface antigens on the 3rd-generation dental pulp stem cells, which had been separated and cultured in the above-described membrane separation culture device, analyzed by flow cytometry. As with dog dental pulp $CD105^+$ cells, the CD105-positive expression rate of the cells separated in the membrane separation culture device was 95.1% in the case of using G-CSF, and it was 89.5% in the case of using SDF-1. In addition, the CD29-, CD44-, CD73-, and CD90-positive expression rates of the cells were 95% or more in both fractions and, thus, it was considered that large quantities of stem cells and/or precursor cells were contained therein. Moreover, the CXCR4-positive expression rate of the cells was a half of that in the case of the dog dental pulp $CD105^+$ cells separated by flow cytometry, and further, the cells were almost negative to CD31 and CD146.

TABLE 1

	membrane-separated dental pulp stem cells	dental pulp $CD105^+$ cells
CD24	1.5%	1.8%
CD29	99.6%	95.9%
CD31	0.2%	0.0%
CD33	6.8%	3.7%
CD34	48.6%	45.5%
CD44	100%	96.2%
CD73	93.3%	97.2%
CD90	92.0%	98.1%
CD105	95.1%	98.5%
CD146	0%	0.8%
CD150	0.6%	2.3%
MHC class I	70.9%	36.0%
MHC class II	3.4%	0.4%
CXCR4	5.3%	12.2%

[0205] Subsequently, using Trizol (Invitrogen), total RNA was separated from the 3rd-generation dental pulp stem cells that had been membrane-separated using G-CSF. Thereafter, first-strand cDNA was synthesized using ReverTra Ace-α (Toyobo), and it was then labeled with Light Cycler-Fast Start DNA master SYBR Green I (Roche Diagnostics). Thereafter, real-time RT-PCR was performed for stem cell markers (CXCR4, Sox2, Stat3, and Bmi1) employing Light Cycler (Roche Diagnostics) in accordance with a program of 95° C.-10 seconds, 62° C.-15 seconds, and 72° C.-8 seconds. Further, as angiogenesis-inducing factors and neurotrophic factors, matrix metalloproteinase (MMP)-3, VEGF-A, granulocyte-monocyte colony-stimulating factor (GM-CSF), NGF, and BDNF were used. As controls, dental pulp $CD105^+$ cells and unseparated dental pulp test cells were used, and primers used as they were standardized with β-actin.

[0206] Table 2 shows primers used in the real-time RT-PCR analysis of stem cell markers, angiogenesis-inducing factors, and neurotrophic factors.

TABLE 2

Canine primers for real-time reverse transcription-polymerase chain reaction

Gene		5'←DNA Sequence→3'	product size	Accession number
Sox2	Forward	AGCTAGTCTCCAAGCGACGA (SEQ ID NO. 1)	193 bp	XM_545216
	Reverse	CCACGTTTGCAACTGTCCTA (SEQ ID NO. 2)		

TABLE 2 -continued

Canine primers for real-time reverse transcription-polymerase chain reaction

Gene		5'←DNA Sequence→3'	product size	Accession number
Bmi1	Forward	CACTCCCGTTCAGTCTCCTC (SEQ ID NO. 3)	150 np	XM_544225
	Reverse	CCAGATGAAGTTGCTGACGA (SEQ ID NO. 4)		
CXCR4	Forward	CTGTGGCAAACTGGTACTTC (SEQ ID NO. 5)	210 bp	NM_001048026
	Reverse	TCAACAGGAGGGCAGGTATC (SEQ ID NO. 6)		
Stat3	Forward	GTGGTGACGGAGAAGCAACA (SEQ ID NO. 7)	191 bp	XM_844672
	Reverse	TTCTGTCTGGTCACCGACTG (SEQ ID NO. 8)		
GM-CSF	Forward	GCAGAACCTGCTTTTCTTGG (SEQ ID NO. 9)	195 bp	S49738
	Reverse	CCCTCAGGGTCAAACACTTC (SEQ ID NO. 10)		
MMPP3	Forward	CCCTCTGATTCCTCCAATGA (SEQ ID NO. 11)	210 bp	AY183143
	Reverse	GGATGGCCAAAATGAAGAGA (SEQ ID NO. 12)		
VEGFA	Forward	CTACCTCCACCATGCCAAGT (SEQ ID NO. 13)	183 bp	NM_001003175
	Reverse	ACGCAGGATGGCTTGAAGAT (SEQ ID NO. 14)		
BDNF	Forward	GTTGGCCGACACTTTTGAAC (SEQ ID NO. 15)	202 bp	NM_001002975
	Reverse	CCTCATCGACATGTTTGCAG (SEQ ID NO. 16)		
GDNF	Forward	GCCGAGCAGTGACTCAAAC (SEQ ID NO. 17)	104 bp	XM_546342
	Reverse	TCTCGGGTGACCTTTTCAG (SEQ ID NO. 18)		
NGF	Forward	CAACAGGACTCACAGGAGCA (SEQ ID NO. 19)	156 bp	XM_540250
	Reverse	ATGTTCACCTCTCCCAGCAC (SEQ ID NO. 20)		
β-actin	Forward	AAGTACCCCATTGAGCACGG (SEQ ID NO. 21)	257 bp	Z70044
	Reverse	ATCACGATGCCAGTGGTGCG (SEQ ID NO. 22)		

[0207] Table 3 shows the expression levels of the mRNAs of stem cell markers, angiogenesis-inducing factors, and neurotrophic factors in the 3rd-generation membrane-separated dog dental pulp stem cells separated using G-CSF and the dental pulp CD105$^+$ cells, analyzed by real time RT-PCR, which were compared with dental pulp test cells. The angiogenesis-inducing factor VEGF and the neurotrophic factor GDNF exhibited almost the same expression levels in the two types of cells. The expression levels of GM-CSF and MMP3 were higher in the membrane-separated cells than in the CD105$^+$ cells by 10 times or more. On the other hand, the expression levels of BDNF and NGF were higher in the CD105-positive cells than in the membrane-separated cells. The expression levels of the stem cell markers CXCR4 and Bmi1 were much higher in the membrane-separated cells than in the CD105$^+$ cells. The expression level of Stat3 was almost the same in the two types of cells, and the expression level of Sox2 was slightly lower than in the membrane-separated cells than in the CD105$^+$ cells.

TABLE 3

	membrane-separated dental pulp stem cells/ unseparated dental pulp test cells	dental pulp CD105$^+$ cells/unseparated dental pulp test cells
Sox2	20.7	64.0
Bmi1	29.9	3.5
CXCR4	8	16.8
Stat3	1.2	0.8
GM-CSF	53.2	5.8
MMP3	313.4	26.1
VEGF	3.8	3.6

TABLE 3-continued

	membrane-separated dental pulp stem cells/ unseparated dental pulp test cells	dental pulp CD105$^+$ cells/unseparated dental pulp test cells
BDNF	1.3	16.0
GDNF	4.1	4.2
NGF	1.8	4.1

Pluripotency In Vitro

[0208] Membrane-separated dental pulp cells were induced to differentiate into blood and nerve for a period from the 3rd generation to the 5th generation. The results are shown in FIG. **9**. As shown in FIG. **9**(*a*), the membrane-separated dental pulp stem cells exhibited ability to differentiate into vascular endothelial cells. In addition, as shown in FIG. **9**(*b*), the membrane-separated dental pulp stem cells exhibited neurosphere formation.

Example 2

Regeneration of Dental Pulp by Autologous Transplantation of Membrane-Separated Dental Pulp Stem Cells into Root Canal after Extirpation of Dental Pulp

[0209] There was established an experimental model, in which the dental pulp was completely removed from the root apex-completely-formed permanent teeth of a dog (Narc, Chiba, Japan), and a cellular fraction was transplanted therein to regenerate dental pulp. The dog was undergone general anesthesia with sodium pentobarbital (Schering-Plough, Ger-

many), and the dental pulp was completely removed from the maxillary second incisor tooth and mandibular third incisor tooth of the dog, and the root apex portion was enlarged to a size of 0.7 mm using #70K-file (MANI, INC., Tochigi, Japan). The membrane-separated dental pulp stem cells of Example 2 were transplanted into the root apex side, and G-CSF was transplanted into the dental crown side. Specifically, the 4th-generation membrane-separated dental pulp stem cells (5×10^5 cells), together with collagen TE (Nitta Gelatin, Osaka, Japan), were labeled with DiI, and they were then autologously transplanted into the lower portion in the root canal. Further, into the upper portion of the root canal, G-CSF (final concentration: 15 ng/μl) together with collagen TE was transplanted. The cavity was treated with zinc phosphate cement (Elite Cement, GC, Tokyo, Japan) and a bonding material (Clearfil Mega Bond, Kuraray), and was then repaired with a composite resin (Clearfil FII, Kuraray, Kurashiki, Japan). As a control, dental pulp CD105-positive cells were used. Fourteen days later, a specimen was prepared. For morphology analysis, the specimen was immobilized with 4% paraformaldehyde (PFA) (Nakarai Tesque, Kyoto, Japan) at 4° C. overnight. Thereafter, the specimen was decalcified with 10% formic acid and was then embedded in paraffin wax (Sigma). A paraffin section (thickness: 5 μm) was stained with hematoxylin-eosin (HE), and was then morphologically observed.

[0210] FIG. **10**(*a*) is a view showing regeneration of the dental pulp by autologous transplantation of G-CSF and membrane-separated dental pulp stem cells. FIG. **10**(*b*) is an enlarged view of an area (b) enclosed with a square in FIG. **10**(*a*). FIG. **10**(*c*) is an enlarged view of an area C enclosed with a square in FIG. **10**(*a*). As shown in FIG. **10**(*a*), FIG. **10**(*b*) and FIG. **10**(*c*), when the membrane-separated dental pulp stem cells are transplanted together with G-CSF, dental pulp-like tissues were formed until the 14th day after completion of the transplantation. As shown in FIG. **10**(*b*), cells in the regenerated tissues have a fusiform or star-like shape, and they were similar to cells in normal dental pulp tissues (FIG. **10**(*d*)). As shown in FIG. **10**(*c*), odontoblast-like cells adhered to the dentin wall of the root canal and extended their projections into canaliculi.

Example 3

Membrane-Separated Human Dental Pulp Stem Cells

[0211] Using a membrane separation culture device (1×10^5 pores/cm^2, pore size: 8 μm), fresh human primary dental pulp cells (1×10^5 cells/100 μl) were dispersed on the upper portion of the membrane. 10 ng/ml or 100 ng/ml G-CSF was added into 10% human serum alone or in DMEM containing 10% human serum in the lower structure of the membrane. Twenty-two hours later, the medium was replaced with fresh medium, the G-CSF was then removed, and the resultant was further cultured in DMEM containing 10% human serum. FIG. **11** shows a phase contrast microscopic image of dental pulp stem cells that have migrated, adhered, and further proliferated. As shown in FIGS. **12**(*a*), (*b*), and (*c*), it became clear that, in all of 10% human serum, 10 ng/ml G-CSF, and 100 ng/ml G-CSF, star-like cells having projections adhered, and that these cells proliferated, as in the case of separating $CD31^-SP$ cells, $CD105^+$ cells or $CXCR4^+$ cells by flow cytometry (FIGS. **12** (*d*) and (*e*)).

Example 4

Membrane-Separated Tissue Stem Cells

[0212] Using our membrane separation culture device (1×10^5 pores/cm^2, pore size: 8 μm), pig dental pulp cells, bone marrow cells, and adipose cells (1×10^5 cells/100 μl) were dispersed on the upper portion of the membrane. Meanwhile, 100 ng/ml G-CSF was added into DMEM containing 10% fetal bovine serum in the lower structure of the membrane. Twenty-two hours later, the medium was replaced with fresh medium, the G-CSF was then removed, and the resultant was further cultured in DMEM containing 10% fetal bovine serum. As shown in FIGS. **13**(*a*), (*b*), and (*c*), it became clear that, in all of the dental pulp, bone marrow, and adipose cells, star-like cells having projections adhered, and that as shown in FIG. **13**(*d*), these cells proliferated, as in the case of separating $CD31^-SP$ cells or $CD105^+$ cells by flow cytometry.

Example 5

1. Comparison of Migration Factors Effective for Migration of Dental Pulp Stem Cells, Analyzed by TAXIScan

[0213] Using TAXIScan-FL (Effector Cell Institute, Tokyo), real-time horizontal chemotaxis analysis was carried out on unseparated human dental pulp cells. A channel optimized to the size of cells (8 μm) was formed between silicone having pores with a pore size of 6 μm and a glass plate, and 1 μl of cells (10^5 cells/ml) was then poured in one side of the channel. Various types of migration factors (10 ng/μl; BDNF, GDNF, NGF, PDGF, G-CSF, SDF-1, bFGF, VEGF, LIF and GM-CSF) were each poured in an amount of 1 μl into the opposite side thereof to form a certain concentration gradient. Based on video images of migration, the number of migrating cells was counted every 3 hours until 15 hours after initiation of the operation. Moreover, using G-CSF and bFGF as migration factors, a change in migration ability in the case of adding 10% fetal bovine serum was examined.

[0214] The measurement results of migration ability in the case of using various types of migration factors are shown in FIG. **14**. Seeing a difference, over time, in migration ability depending on various migration factors, large quantities of migrating cells were observed when BDNF, GDNF, NGF, PDGF and G-CSF were used. In the case of using SDF-1, bFGF and LIF as well, relatively large quantities of migrating cells were observed. In the case of using GM-CSF, however, migration of cells was hardly observed. As such, a comparative review was made between the migration factors G-CSF and bFGF, whose agents satisfying clinically used standards (Good Manufacturing Practice (GMP)) can be easily obtained and which had already received pharmaceutical approval in Japan.

[0215] The measurement results of migration ability obtained using G-CSF and bFGF are shown in FIG. **15**. When G-CSF and bFGF were added, the migration ability of cells became higher than that in the case of adding only 10% serum. When 10% serum was added to each of G-CSF and bFGF, the migration of the cells was further promoted. In particular, it became clear that G-CSF promotes the migration of cells more strongly than bFGF does. From these results, it was found that it is effective in carrying out membrane separation of migrating cells by adding 10% serum to G-CSF.

[0216] As described above, with regard to the expression of a stem cell marker, the membrane-separated cells that had been separated using 10 ng/ml G-CSF had a stem cell marker expression rate similar to that of the $CD105^+$ cells separated by flow cytometry. The membrane-separated cells that had been separated using 100 ng/ml G-CSF had the expression rates of CXCR4 and G-CSFR that were higher than those of the $CD105^+$ cells and, thus, it has been suggested that the membrane-separated cells might contain larger quantities of stem cells. Moreover, the membrane-separated cells that had been separated using 100 ng/ml G-CSF had cell proliferative ability and migration ability that were higher than those of the $CD105^+$ cells. Furthermore, with regard to the expression of mRNAs of angiogenic factors and neurotrophic factors as well, the membrane-separated cells separated using 100 ng/ml G-CSF exhibited the highest expression levels. We had already reported that dental pulp $CD105^+$ cells have high angiogenesis-inducing ability, high nerve-inducing ability, and high ability to regenerate the dental pulp. However, it has been suggested from the results of this experiment that the membrane-separated cells separated using 100 ng/ml G-CSF are also effective to regenerate the dental pulp/dentin, as in the case of the $CD105^+$ cells.

2. Number of Cells Dispersed for Separation of Dental Pulp Stem Cells Using Membrane Separation Device

[0217] Cellculture Insert (polycarbonate base material membrane (Polycarbonate Membrane) Transwell (registered trademark) Inserts; 2×10^5 pores/cm^2, pore size: 8 μm, diameter of bottom surface: 6.4 mm, diameter of opening portion: 11.0 mm, height: 17.5 mm) (Corning), used an upper structure, was inserted into a 24-well plate (diameter: 15.0 mm, diameter of opening portion: 15.0 mm, height: 22.0 mm) (Falcon), used as a lower structure, and the thus prepared device was used as a membrane separation device. To impart a non-cell-adhesive property to the polycarbonate base material membrane, the membrane had previously been immersed in an aqueous solution prepared by adding 0.1% ethanol to a 1000 ppm aqueous solution of a polyvinyl pyrrolidone-polyvinyl acetate copolymer (vinyl pyrrolidone/vinyl acetate (6/4) copolymer ("Kollidon VA64," manufactured by BASF)) to seal it. Thereafter, the resulting membrane was modified by irradiation with a γ-ray (25 kGy), thereby preparing a separation membrane. As a result of the aforementioned operation, the polyvinyl pyrrolidone-polyvinyl acetate copolymer was bound to the surface of the base material membrane via a covalent bond. However, the polymer had high safety and, also, the ethanol used simultaneously with the copolymer was decomposed by irradiation with the γ-ray and the concentration thereof was reduced. Accordingly, this surface modification was highly safe. The 2nd-generation human dental pulp cells were dispersed on the upper portion of this separation membrane at a cell density of 2×10^4 cells/100 μl, 1×10^5 cells/100 μl. Meanwhile, G-CSF (final concentration: 100 ng/ml) was added into Dulbecco's modified Eagle's medium (DMEM) containing 10% human serum in 24 wells of the lower structure of the membrane. Forty-eight hours later, the G-CSF was removed, and the medium was replaced with another DMEM containing 10% human serum. Thereafter, the number of cells adhered to the lower portions of 24 wells was measured under phase contrast microscope.

[0218] As a result, the separated cell percentage was 5% at a cell density of 2×10^4 cells/100 μl, and was 1% at a cell density of 1×10^5 cells/100 μl. These results suggested that cell separation efficiency be different depending on the number of cells.

3. Action Time of Migration Factors for Separation of Dental Pulp Stem Cells Using Membrane Separation Device

[0219] The 2nd-generation human dental pulp cells were dispersed on the upper portion of the separation membrane of a membrane separation device at a cell density of 2×10^4 cells/100 μl. Meanwhile, G-CSF (final concentration: 100 ng/ml) was added into DMEM containing 10% human serum in 24 wells of the lower structure of the membrane. Thereafter, 12, 24, 48, and 72 hours later, the number of cells adhered to the lower portions of the 24 wells was counted under a phase contrast microscope.

[0220] The action times of G-CSF were set at 12, 24, 48, and 72 hours, and the number of adhering cells was counted in each time point. As a result, 12 hours later, the separated cell percentage was 0.3%, 24 hours later, it was 1.7%, 48 hours later, it was 5%, and 72 hours later, it was also 5%.

4. Concentration of G-CSF for Separation of Dental Pulp Stem Cells Using Membrane Separation Device

[0221] The 2nd-generation human dental pulp cells were dispersed on the upper portion of the separation membrane of a membrane separation device at a cell density of 2×10^4 cells/100 μl. Meanwhile, G-CSF was added into Dulbecco's modified Eagle's medium (DMEM) containing 10% human serum in 24 wells of the lower structure of the membrane to final concentrations of 0, 10, 100, and 500 ng/ml. Forty-eight hours later, the G-CSF was removed, and the medium was then replaced with another DMEM containing 10% human serum. Then, the number of cells adhered to the lower portions of the 24 wells was counted under phase contrast microscope. Thereafter, the cells were further cultured, and after they had become 70% confluent, they were subcultured.

[0222] The above described dental pulp stem cells, which had been membrane-separated using various concentrations of G-CSF, were subcultured to the 6th generation. Thereafter, the expression rates of the stem cell surface antigen markers were measured by flow cytometry. Specifically, the cells were dispersed in DMEM containing 2% serum at a cell density of 1×10^6 cells/ml, and were then labeled with stem cell marker antibodies at 4° C. for 30 minutes. Specifically, the cells were labeled at 4° C. for 90 minutes using mouse IgG1 negative control (AbD Serotec Ltd.), hamster IgG (PE-cy7) (eBio299Arm) (eBioscience), rat IgG2b (PE-cy7) (RTK4530) (Biolegend), mouse IgG1 (APC) (NOPC-21) (Biolegend), mouse IgG1 (RPE) (SFL928PE) (AbD Serotec), mouse IgG1 (Alexa647) (F8-11-13) (AbD Serotec), mouse IgG2a (FITC) (S43.10) (MACS), mouse IgG1 (FITC) (MOPC-21) (Biolegend), antibodies to the following: CD29 (Phycoerythrin, PE-cy7) (eBio299Arm) (eBioscience), CD31 (PE) (WM59) (BD Pharmingen), CD44 (PE-cy7) (IM7) (eBioscience), CD73 (APC) (AD2) (Biolegend), CD90 (Alexa647) (F15-42-1) (AbD Serotec), CD105 (PE) (43A3) (Biolegend), CD146 (Alexa647) (OJ79c) (AbD Serotec), CXCR4 (FITC) (12G5) (R&D Systems), G-CSFR (CD114) (FITC) (38660). As controls, human dental pulp $CD105^+$ cells separated by flow cytometry and unseparated human dental pulp test cells were used.

[0223] Subsequently, using Trizol (Invitrogen), total RNA was separated from the 6th-generation membrane-separated cells separated using various concentrations of G-CSF. Thereafter, first-strand cDNA was synthesized using Rever-Tra Ace-α (Toyobo), and it was then labeled with Light Cycler-Fast Start DNA master SYBR Green I (Roche Diagnostics). Thereafter, real-time RT-PCR was performed for angiogenesis-inducing factors and neurotrophic factors, employing Light Cycler (Roche Diagnostics), in accordance with a program of 95° C.-10 seconds, 65° C.-15 seconds, and 72° C.-8 seconds. As angiogenesis-inducing factors and neurotrophic factors, the primers of granulocyte-monocyte colony-stimulating factor (GM-CSF), matrix metalloproteinase (MMP)-3, VEGF-A, brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and neurotrophin-3 (NT-3) were used (Table 4). As controls, dental pulp $CD105^+$ cells and unseparated human dental pulp test cells were used, and they were standardized with β-actin.

TABLE 4

Gene		5'←DNA Sequence→3'	product size	Accession number
Oct4	Forward	CAGTGCCCGAAACCCACAC (SEQ ID NO. 23)	161	NM_002701
	Reverse	GGAGACCCAGCAGCCTCAAA (SEQ ID NO. 24)		
Nanog	Forward	CAGAAGGCCTCAGCACCTAC (SEQ ID NO. 25)	111	NM_024865
	Reverse	ATTGTTCCAGGTCTGGTTGC (SEQ ID NO. 26)		
Sox2	Forward	AATGCCTTCATGGTGTGGTC (SEQ ID NO. 27)	203	NM_003106
	Reverse	CGGGGCCGGTATTTATAATC (SEQ ID NO. 28)		
Rex1	Forward	TGGACACGTCTGTGCTCTTC (SEQ ID NO. 29)	168	BC032244
	Reverse	CTCGAACCTTCCAGATCACC (SEQ ID NO. 30)		
Stat3	Forward	GTGGTGACGGAGAAGCAGCA (SEQ ID NO. 31)	191	NM_139276
	Reverse	TTCTGCCTGGTCACTGACTG (SEQ ID NO. 32)		
CXCR4	Forward	CCGTGGCAAACTGGTACTTT (SEQ ID NO. 33)	210	NM_001008540
	Reverse	TCAGCAGGAGGGCAGGGATC (SEQ ID NO. 34)		
GM-CSF	Forward	GCCTGGAGCTGTACAAGCAG (SEQ ID NO. 35)	193 bp	NM_000758
	Reverse	CAGCAGTCAAAGGGGATGAC (SEQ ID NO. 36)		
MMP3	Forward	CCTCAGGAAGCTTGAACCTG (SEQ ID NO. 37)	192 bp	NM_002422
	Reverse	GGGAAACCTAGGGTGTGGAT (SEQ ID NO. 38)		
VEGFA	Forward	ATGGCAGAAGGAGACCAGAA (SEQ ID NO. 39)	224 bp	NM_001033756
	Reverse	ATGGCGATGTTGAACTCCTC (SEQ ID NO. 40)		
BDNF	Forward	AAACATCCGAGGACAAGGTG (SEQ ID NO. 41)	202 bp	NM_170735
	Reverse	CGTGTACAAGTCTGCGTCCT (SEQ ID NO. 42)		
GDNF	Forward	CCAACCCAGAGAATTCCAGA (SEQ ID NO. 43)	150 bp	NM_000514
	Reverse	AGCCGCTGCAGTACCTAAAA (SEQ ID NO. 44)		
NGF	Forward	ATACAGGCGGAACCACACTC (SEQ ID NO. 45)	181 bp	NM_002506
	Reverse	GCCTGGGGTCCACAGTAAT (SEQ ID NO. 46)		
NT-3	Forward	AGACTCGCTCAATTCCCTCA (SEQ ID NO. 47)	187 bp	BC107075
	Reverse	GGTGTCCATTGCAATCACTG (SEQ ID NO. 48)		
β-actin	Forward	GGACTTCGAGCAAGAGATGG (SEQ ID NO. 49)	234 bp	NM_001101
	Reverse	AGCACTGTGTTGGCGTACAG (SEQ ID NO. 50)		

[0224] Furthermore, the 7th-generation membrane-separated dental pulp cells were induced to differentiate into blood, nerve, adipose, dentin, and bone in vitro according to an ordinary method. Further, membrane-separated human cells that had been separated with various concentrations of G-CSF were compared with one another, in terms of cell proliferative ability by stimulation with 10% human serum or 100 ng/ml G-CSF, and cell migration ability with 10% human serum or 100 ng/ml G-CSF.

[0225] Dental pulp stem cells, which had adhered to the plate and had further proliferated, were observed from phase contrast microscopic images. With regard to unseparated 6^{th}-generation human dental pulp test cells, 6th-generation human dental pulp $CD105^+$ cells, membrane-separated cells that had been membrane-separated only with 10% serum and had been then cultured for 7 days, membrane-separated cells that had been membrane-separated with 10 ng/ml G-CSF+10% serum and had been then cultured for 7 days, membrane-separated cells that had been membrane-separated with 100 ng/ml G-CSF+10% serum and had been then cultured for 7 days, and membrane-separated cells that had been membrane-separated with 500 ng/ml G-CSF+10% serum and had been then cultured for 7 days, phase contrast microscopic images were obtained. Even in a case in which any of the

concentrations such as 10% human serum, or 0 ng/ml, 10 ng/ml, 100 ng/ml or 500 ng/ml G-CSF, is used, adhesion and proliferation of star-like cells having projections were observed. The number of adhering cells was counted. As a result, in the case of 100 ng/ml G-CSF, the separated cell percentage was 5%, and then, in the case of 500 ng/ml G-CSF, it was 4%, in the case of 10 ng/ml G-CSF, it was 3%, and in the case of 0 ng/ml G-CSF, it was 2%.

[0226] Subsequently, the analysis of stem cell surface markers by flow cytometry is shown in Table 5. The stem cell marker expression rates were compared by flow cytometry. As a result, CD29, CD44, CD73, and CD90 were all positive, and no difference was found. The expression rate of CD105 was 19% in unseparated human dental pulp test cells, but it was 90% or more in the membrane-separated cells separated with 10 ng/ml and 100 ng/ml G-CSF, as in the case of the control $CD105^+$ cells. Moreover, in the membrane-separated cells separated with 500 ng/ml and 0 ng/ml G-CSF, the expression rates of CD105 were low levels, which were 67% and 58%, respectively. The expression rate of CXCR4 was 4.5% in unseparated human dental pulp test cells, was 8% in the $CD105^+$ cells, and was 5% in the membrane-separated cells separated with 0 ng/ml G-CSF, which were all low levels. In contrast, the expression rate of CXCR4 was highest (15%) in the membrane-separated cells separated with 100 ng/ml G-CSF, and was 10% or more in the membrane-separated cells separated with 10 ng/ml and 500 ng/ml G-CSF. Furthermore, the expression rate of G-CSFR as a G-CSF receptor was 18% in $CD105^+$ cells, and was the highest (76%) in the membrane-separated cells separated with 100 ng/ml G-CSF. Thus, a reduction in the expression rate was found in the order of 500 ng/ml G-CSF and 10 ng/ml G-CSF. From these results, it has been suggested that membrane-separated cells separated with 100 ng/ml G-CSF, in which the positive expression rates of CD105, CXCR4, and G-CSFR are the highest, might contain the greatest quantities of stem cells and/or precursor cells.

[0227] The pluripotency of membrane-separated cells that had been separated using 100 ng/ml G-CSF was studied. Specifically, the angiogenesis-inducing ability and nerve-inducing ability of the membrane-separated cells that had been separated using G-CSF (concentration: 100 ng/ml) were examined based on a phase contrast microscopic image. As in the case of $CD105^+$ cells, 6 hours later, the membrane-separated cells formed a cord-like structure on matrigel, and thus, the cells showed ability to differentiate into vascular endothelial cells. In the case of unseparated human dental pulp test cells, such formation of a cord-like structure was not seen even if it was observed for a long period of time. Moreover, the membrane-separated cells separated with 100 ng/ml G-CSF were found to form neurospheres on the 14th day of induction, as in the case of $CD105^+$ cells, although such formation of neurospheres was hardly seen in unseparated human dental pulp test cells. The adipose-inducing ability of the membrane-separated cells separated with G-CSF (concentration: 100 ng/ml) was examined based on an optical microscopic image. Adipose induction was observed in all of cellular fractions. The expression level of adipose marker mRNA was higher in these membrane-separated cells than in unseparated human dental pulp test cells. The bone/dentin-inducing ability of the membrane-separated cells separated with G-CSF having an extremely preferred concentration (100 ng/ml) was examined based on an optical microscopic image. Such bone/dentin induction was also observed in all of cellular fractions. The expression level of bone/dentin marker mRNA was lower in the membrane-separated cells than in unseparated human dental pulp test cells.

[0228] The results obtained by analyzing the expression of mRNAs of angiogenesis-inducing factors and neurotrophic factors by real-time RT-PCR are shown in Table 6.

TABLE 5

		membrane-separated cells				
	unseparated dental pulp test cells	G-CSF 0 ng/ml	G-CSF 10 ng/ml	G-CSF 100 ng/ml	G-CSF 500 ng/ml	dental pulp $CD105^+$ cells
CD29	94.9%	97.8%	94.9%	96.9%	97.4%	95.6%
CD31	0.0%	0.0%	0.2%	0.0%	0.3%	0.4%
CD44	97.2%	98.3%	98.0%	94.8%	94.8%	94.1%
CD73	99.0%	90.6%	99.5%	99.2%	99.3%	97.1%
CD90	99.4%	97.6%	99.5%	99.4%	99.0%	99.6%
CD105	18.9%	58.3%	94.0%	98.1%	66.9%	96.8%
CD146	14.6%	13.3%	16.2%	9.2%	16.7%	13.0%
CXCR4	4.5%	5.1%	12.1%	15.3%	10.2%	7.8%
G-CSFR	9.3%	14.5%	28.6%	75.9%	32.5%	18.0%

TABLE 6

		membrane-separated cells				
	unseparated dental pulp test cells	G-CSF 0 ng/ml	G-CSF 10 ng/ml	G-CSF 100 ng/ml	G-CSF 500 ng/ml	dental pulp $CD105^+$ cells
Oct4	1.0	1.1	1.3	2.0	1.3	1.6
Nanog	1.0	1.1	1.3	1.9	1.3	2.1

TABLE 6-continued

		membrane-separated cells				
	unseparated dental pulp test cells	G-CSF 0 ng/ml	G-CSF 10 ng/ml	G-CSF 100 ng/ml	G-CSF 500 ng/ml	dental pulp $CD105^+$ cells
Sox2	1.0	0.7	26.0	27.7	19.6	40.8
Rex1	1.0	1.7	2.8	3.0	1.5	1.6
Stat3	1.0	0.7	1.3	1.9	1.2	1.0
CXCR4	1.0	1.5	21.6	35.5	32.2	42.8
GM-CSF	1.0	1.2	42.2	57.7	50.9	52.3
MMP3	1.0	4.5	40.0	64.9	60.5	40.3
VEGFA	1.0	0.5	4.0	6.2	5.0	3.7
BDNF	1.0	1.2	2.0	7.5	4.4	3.7
GDNF	1.0	0.8	3.2	4.6	4.0	2.5
NGF	1.0	0.7	1.9	3.1	2.5	1.8
NT-3	1.0	1.0	3.0	4.2	3.8	3.1

[0229] The expression levels of the angiogenesis-inducing factor/neurotrophic factor GM-CSF and MMP3 were higher in the membrane-separated cells than in the unseparated human dental pulp test cells by 10 times or more, regardless of the concentration of G-CSF. The expression levels of VEGF, BDNF, GDNF, NGF and NT-3 in the membrane-separated cells were almost the same levels or 2 times greater than those in $CD105^+$ cells (in the case of the membrane-separated cells separated with 100 ng/ml G-CSF), and were higher than those in the unseparated human dental pulp test cells. The expression level of the stem cell marker Sox2 was higher in the membrane-separated cells than in the unseparated cells by 10 times or more. The expression levels of Oct4, Nanog and Rex1 in the membrane-separated cells were almost the same levels or 2 times greater than $CD105^+$ cells (in the case of the membrane-separated cells separated with 100 ng/ml G-CSF).

[0230] A graph showing a comparison regarding the cell proliferative ability of membrane-separated cells to human serum ($^*p<0.05$) is shown in FIG. **16**. In terms of the proliferative ability to serum, no significant difference was found in all of cellular fractions. A comparison regarding the cell proliferative ability of membrane-separated cells, in which G-CSF (100 ng/ml) was used ($^{**}p<0.01$, $^*p<0.05$: vs unseparated human dental pulp test cells, $^{\#\#}p<0.01$: vs dental pulp $CD105^+$ cells), is shown in FIG. **17**. The proliferative ability to G-CSF of the membrane-separated cells separated with 100 ng/ml and 500 ng/ml G-CSF was the highest and, thus, the membrane-separated cells had a significant difference from both the unseparated human dental pulp test cells and $CD105^+$ cells. A comparison regarding the cell migration ability of membrane-separated cells to G-CSF having different concentrations ($^{**}p<0.01$, $^*p<0.05$: vs unseparated human dental pulp test cells, $^{\#}p<0.01$, $^{\#}p<0.05$: vs dental pulp $CD105^+$ cells) is shown in FIG. **18**. The migration ability to G-CSF of the membrane-separated cells separated with 100 ng/ml G-CSF was highest, and thus, the membrane-separated cells had a significant difference from both the unseparated human dental pulp test cells and CD 105^+ cells.

5. Angiogenesis-Inducing Ability of Membrane-Separated Cells Examined In Vivo Using Mouse Lower Limb Ischemia Model

[0231] A mouse lower limb ischemia model was produced, and into its ischemic site, unseparated human dental pulp test cells, membrane-separated cells separated with 100 ng/ml G-CSF, and $CD105^+$ cells were each transplanted. Fourteen days later, blood flow was analyzed by laser Doppler technique. With regard to angiogenesis, frozen sections were produced and then subjected to immunohistological analysis via BS-1 lectin staining

[0232] As a result of the laser Doppler analysis, it was found that blood flow was not improved so much by transplantation of the test cells, but that blood flow was significantly improved by transplantation of the membrane-separated cells, as in the case of the transplantation of the $CD105^+$ cells. Moreover, when frozen sections were produced and were then stained with BS-1 lectin, angiogenesis was observed by transplantation of the membrane-separated cells, as in the case of transplantation of $CD105^+$ cells.

6. Ability of Membrane-Separated Cells to Regenerate Dental Pulp Examined In Vivo Using SCID Mouse

[0233] An extracted human tooth was sliced, and one end thereof was then sealed with cement. Thereafter, unseparated human dental pulp test cells, membrane-separated cells separated with 100 ng/ml G-CSF, or $CD105^+$ cells were used as Scaffold, and the cells were injected into the tooth section together with collagen. The resulting tooth section was transplanted into the subcutis of an SCID mouse. Three weeks later, the above three types of cells were compared with one another in terms of ability to regenerate the dental pulp. The morphology was analyzed by HE staining. The nerve regeneration ability and angiogenesis-inducing ability were immunohistologically analyzed by PGP9.5, BS1 lectin staining and Ki67 staining Localization of the transplanted cells was analyzed by in situ hybridization performed on the human-specific gene Alu. Moreover, the expression level of the dental pulp-specific marker mRNA in the regenerated dental pulp tissues was compared with that in normal dental pulp tissues.

[0234] As a result of the HE staining, formation of dental pulp-like tissues was observed by transplantation of the membrane-separated human cells, as in the case of transplantation of $CD105^+$ cells. On the other hand, by transplantation of the unseparated test cells, only a low level of formation of dental pulp-like tissues was observed. In addition, as a result of the BS1 lectin and PGP9.5 staining, angiogenesis and regeneration of nerves were observed by transplantation of the membrane-separated cells, as in the case of transplantation of $CD105^+$ cells. Moreover, proliferation of the transplanted cells was hardly observed. As a result of the in situ hybridization performed on Alu, it became clear that the host mouse cells form the regenerated dental pulp-like tissues.

[0235] The results obtained by examining at an mRNA expression level that the regenerated dental pulp-like tissues are dental pulp are shown in Table 7.

TABLE 7

		regenerated dental pulp tissue	
	mouse normal dental pulp tissue	unseparated test cells transplantation	membrane-separated cells transplantation
Syndecan 3	1.0	0.7	1.5
Tenascin C	1.0	0.2	0.7
TRH-DE	1.0	0.7	1.8
Periostin	1.0	5.9	0.9
aP2	—	—	—
Runx 2	1.0	0.1	0.2
Enamelysin	1.0	0.0	0.0

[0236] The expression level of TRH-DE serving as a dental pulp-specific marker and the expression levels of Syndecan and Tenascin C that are reportedly expressed at high levels in the dental pulp in the regenerated dental pulp-like tissues were the same levels as those in normal dental pulp tissues. On the other hand, the expression of periodontium, adipose tissue, and bone/dentin marker mRNAs was not observed in the regenerated dental pulp-like tissues. From these results, it became clear that the regenerated dental pulp-like tissues obtained by transplantation of the membrane-separated cells are dental pulp.

Example 6

Method for Separating Dental Pulp, Bone Marrow, and Adipose Stem Cells by Membrane

1. Separation of Dental Pulp, Bone Marrow, and Adipose Stem Cells Using Membrane Separation Device

[0237] Whether stem cells can also be separated from the bone marrow or adipose cells by a membrane separation method, as in the case of dental pulp cells, was examined. As a membrane separation device, Cellculture Insert (polycarbonate base material membrane (Polycarbonate Membrane) Transwell (registered trademark) Inserts; 2×10^5 pores/cm^2, pore size: 8 μm, diameter of bottom surface: 6.4 mm, diameter of opening portion: 11.0 mm, height: 17.5 mm) (Corning), used an upper structure, was inserted into a 24-well plate (diameter: 15.0 mm, diameter of opening portion: 15.0 mm, height: 22.0 mm) (Falcon), used as a lower structure, and the thus prepared device was used as a membrane separation device. To avoid cell adhesion, the surface of the polycarbonate base material membrane was subjected to a coating treatment. To impart a non-cell-adhesive property to the polycarbonate base material, the surface of the polycarbonate base material membrane had previously been modified by immersing it in a treating aqueous solution prepared by adding 0.1% ethanol to a 1000 ppm aqueous solution of a polyvinyl pyrrolidone-polyvinyl acetate copolymer (vinyl pyrrolidone/vinyl acetate (6/4) copolymer ("Kollidon VA64," manufactured by BASF)) to seal it, irradiating the resulting membrane with a γ-ray (25 kGy), thereby preparing a separation membrane. Thereafter, the pig dental pulp, bone marrow, and adipose cells at the 2nd passage of culture were each dispersed on the upper portion of this separation membrane at a cell density of 1.5×10^4 cells/100 μl. Meanwhile, G-CSF (final concentration: 100 ng/ml) was added into Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS in 24 wells of the lower structure of the membrane. Twenty-four hours later, the G-CSF was removed, and the medium was replaced with another DMEM containing 10% FBS, followed by performing a culture. After the cells had become 70% confluent, they were subcultured.

[0238] As a result, we found that bone marrow-derived and adipose stem cells are also separated in the lower portion of the separation membrane under the same conditions as those applied to separation of the dental pulp stem cells.

2. Measurement of Positive Expression Rates of Stem Cell Surface Antigen Markers

[0239] Membrane-separated pig dental pulp, bone marrow, and adipose cells were subcultured for five generations, and the positive expression rates of stem cell surface antigen markers were then measured. Thereafter, the cells were dispersed in PBS containing 2% FBS at a cell density of 1×10^6 cells/ml. After that, the cells were labeled with stem cell marker antibodies at 4° C. for 60 minutes, followed by performing flow cytometry. Specifically, the cells were labeled at 4° C. for 60 minutes using mouse IgG1 negative control (AbD Serotec Ltd.), hamster IgG (PE-cy7) (eBio299Arm) (eBioscience), rat IgG2b (PE-cy7) (RTK4530) (Biolegend), mouse IgG1 (APC) (NOPC-21) (Biolegend), mouse IgG1 (RPE) (SFL928PE) (AbD Serotec), mouse IgG1 (Alexa647) (F8-11-13) (AbD Serotec), mouse IgG2a (FITC) (S43.10) (MACS), mouse IgG1 (FITC) (MOPC-21) (Biolegend), antibodies to the following: CD29 (Phycoerythrin, PE-cy7) (eBioHMbl-1) (eBioscience), CD31 (PE) (LCI-4) (AbD Serotec), CD44 (PE-cy7) (IM7) (eBioscience), CD73 (APC) (AD2) (Biolegend), CD90 (Alexa647) (F15-42-1) (AbD Serotec), CD105 (FITC) (MEM-229) (Abcam), CXCR4 (FITC) (12G5) (R&D Systems), G-CSFR(CD114) (Alexa 488) (S1390) (Abcam). After completion of the labeling, Hank's buffer, to which Hepes had been added to a final concentration of 0.01 M and FBS had also been added to a final concentration of 2%, was added to the resulting cells, followed by performing flow cytometry. As negative controls, unseparated dental pulp, bone marrow, and adipose test cells were used.

[0240] The comparative results regarding the expression rates of the stem cell markers, obtained by flow cytometry, are shown in Table 8.

TABLE 8

	dental pulp		bone marrow		adipose	
	unseparated test cells	membrane-separated cells	unseparated test cells	membrane-separated cells	unseparated test cells	membrane-separated cells
CD29	99.5%	96.7%	93.6%	94.7%	94.3%	92.4%
CD44	97.2%	96.0%	92.8%	93.7%	99.2%	97.3%
CD73	92.6%	91.0%	90.6%	91.7%	90.1%	90.1%

TABLE 8-continued

	dental pulp		bone marrow		adipose	
	unseparated test cells	membrane-separated cells	unseparated test cells	membrane-separated cells	unseparated test cells	membrane-separated cells
CD90	94.8%	92.4%	90.3%	90.9%	90.7%	90.4%
CD105	14.7%	70.8%	22.3%	73.2%	25.9%	61.7%
CXCR4	5.9%	14.1%	4.1%	14.2%	2.8%	7.0%
G-CSFR	23.7%	74.2%	21.9%	48.5%	23.6%	49.5%

[0241] As a result, CD29, CD44, CD73, and CD90 were almost positively expressed, and there were found no differences between the membrane-separated cells and the unseparated test cells. The positive expression rates of CD105 in unseparated dental pulp, bone marrow, and adipose test cells were 14.7%, 22.3%, and 25.9%, respectively. On the other hand, the positive expression rates of CD105 in the membrane-separated dental pulp, bone marrow, and adipose cells were 70.8%, 73.2%, and 61.7%, respectively. Thus, the positive expression rate of CD105 was higher in the membrane-separated cells than in the unseparated test cells, regardless of the types of the cells. Moreover, the positive expression rates of CXCR4 in unseparated dental pulp, bone marrow, and adipose test cells were 5.9%, 4.1%, and 2.8%, respectively. On the other hand, the positive expression rates of CXCR4 in the membrane-separated dental pulp, bone marrow, and adipose cells were 14.1%, 14.2%, and 7.0%, respectively, thereby showing higher positive expression rates. Furthermore, in the case of G-CSFR as a G-CSF receptor as well, the positive expression rates of G-CSFR in unseparated dental pulp, bone marrow, and adipose test cells were 23.7%, 21.9%, and 23.6%, respectively, whereas the positive expression rates of G-CSFR in the membrane-separated dental pulp, bone marrow, and adipose cells were 74.2%, 48.5%, and 49.5%, respectively, showing higher positive expression rates. From these results, it was found that stem cells/precursor cells, in which the positive expression rates of CD105, CXCR4 and G-CSFR are high, can be separated according to a membrane separation method, not only from dental pulp cells, but also from bone marrow cells and adipose cells.

3. Analysis of Expression of mRNAs of Angiogenic Factors, Neurotrophic Factors, and Stem Cell markers

[0242] Subsequently, the expression of the mRNAs of angiogenic factors, neurotrophic factors, and stem cell markers was analyzed by real-time RT-PCR. Using Trizol (Invitrogen), total RNA was extracted from each of the membrane-separated dental pulp, bone marrow and adipose cells, which had been subcultured for 5 generations, and also from each of the unseparated dental pulp, bone marrow and adipose cells, which had been subcultured for 5 generations. The thus extracted total RNA was treated with DNase (Roche), and thereafter, first-strand cDNA was synthesized therefrom using ReverTra Ace-α (TOYOBO). The synthesized cDNA was labeled with Light Cycler-Fast Start DNA master SYBR Green I (Roche Diagnostics). Thereafter, real-time RT-PCR was performed for angiogenic factors, neurotrophic factors, and stem cell markers, employing Light Cycler (Roche Diagnostics) in accordance with a program of 95° C.-10 seconds, 65° C. or 60° C.-15 seconds, and 72° C.-8 seconds. Primers used as such angiogenic factors, neurotrophic factors, and stem cell markers are shown in Table 9. The primers of granulocyte-monocyte colony-stimulating factor (GM-CSF), vascula endothelin growth factor (VEGF)-A, matrix metalloproteinase (MMP)-3, chemokine (C-X-C motif) receptor (CXCR)-4, brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), nerve growth factor (NGF), nanog homeobox (Nanog), SRY (sex determining region Y)-box 2 (Sox2), signal transducer and activator of transcription (STAT)-3, telomerase reverse transcriptase (Tert), Bmi1 polycomb ring finger oncogene (Bmi-1) were standardized with β-actin.

TABLE 9

Gene		5'←DNA Sequence→3'	product size	Accession number
Nanog	Forward	CCCCGAAGCATCCATTTCC (SEQ ID NO. 51)	101 bp	DQ447201
	Reverse	CGAGGGTCTCAGCAGATGACAT (SEQ ID NO. 52)		
Sox2	Forward	AATGCCTTCATGGTGTGGTC (SEQ ID NO. 53)	203 bp	DQ400923
	Reverse	CGGGGCCGGTATTTATAATC (SEQ ID NO. 54)		
STAT3	Forward	GTGGTGACAGAGAAGCAGCA (SEQ ID NO. 55)	191 bp	NM_001044580
	Reverse	TTCTGCCTGGTCACTGACTG (SEQ ID NO. 56)		
Tert	Forward	CAGGTGTACCGCCTCCTG (SEQ ID NO. 57)	180 bp	DQ400924
	Reverse	CCAGATGCAGTCTTGCACTT (SEQ ID NO. 58)		
Bmi-1	Forward	ATATTTACGGTGCCCAGCAG (SEQ ID NO. 59)	179 bp	
	Reverse	GAAGTGGCCCATTCCTTCTC (SEQ ID NO. 60)		
CXCR4	Forward	CCGTGGCAAACTGGTACTTT (SEQ ID NO. 61)	209 bp	NM_213773
	Reverse	TCAACAGGAGGGCAGGTATC (SEQ ID NO. 62)		
GM-CSF	Forward	GCCCTGAGCCTTCTAAACAA (SEQ ID NO. 63)	193 bp	AY116504
	Reverse	GTGCTGCTCATAGTGCTTGG (SEQ ID NO. 64)		

TABLE 9 -continued

Gene		5'←DNA Sequence→3'	product size	Accession number
MMP3	Forward	ACCCAGATGTGGAGTTCCTG (SEQ ID NO. 65)	171 bp	NM_001166308
	Reverse	GGAGTCACTTCCTCCCAGATT (SEQ ID NO. 66)		
VEGFA	Forward	ATGGCAGAAGGAGACCAGAA (SEQ ID NO. 67)	224 bp	NM_214084
	Reverse	ATGGCGATGTTGAACTCCTC (SEQ ID NO. 68)		
BDNF	Forward	TTCAAGAGGCCTGACATCGT (SEQ ID NO. 69)	180 bp	NM_214259
	Reverse	AGAAGAGGAGGCTCCAAAGG (SEQ ID NO. 70)		
GDNF	Forward	ACGGCCATACACCTCAATGT (SEQ ID NO. 71)	111 bp	XM_003133897
	Reverse	CCGTCTGTTTTTGGACAGGT (SEQ ID NO. 72)		
NGF	Forward	TGGTGTTGGGAGAGGTGAAT (SEQ ID NO. 73)	210 bp	XM_003355233
	Reverse	CCGTGTCGATTCGGATAAA (SEQ ID NO. 74)		
β-actin	Forward	CTCTTCCAGCCCTCCTTCCT (SEQ ID NO. 75)	80 bp	AJ312193
	Reverse	ACGTCGCACTTCATGATCGA (SEQ ID NO. 76)		

[0243] The results obtained by analyzing the expression of mRNAs of the angiogenesis-inducing factors and the neurotrophic factors are shown in Table 10. The expression levels of GM-CSF, MMP3, and BDNF were higher in the membrane-separated cells than in the unseparated test cells by approximately 5 to 10 times. On the other hand, the expression levels of VEGF, GDNF, and NGF in the membrane-separated cells were almost the same levels or 2 times greater than those in $CD105^+$ cells (in the case of the membrane-separated cells separated with 100 ng/ml G-CSF), and were higher in the membrane-separated cells than in the unseparated dental pulp test cells.

TABLE 10

	dental pulp		bone marrow		adipose	
	unseparated test cells	membrane-separated cells	unseparated test cells	membrane-separated cells	unseparated test cells	membrane-separated cells
Nanog	1.0	2.3	0.3	1.2	0.3	0.5
Sox2	1.0	17.3	0.5	1.4	0.2	0.4
STAT3	1.0	2.2	0.7	1.3	0.4	0.5
GM-CSF	1.0	6.3	0.7	3.9	0.3	0.4
VEGF	1.0	2.1	0.4	0.5	0.2	0.4
MMP3	1.0	10.9	0.6	2.1	0.4	0.7
CXCR4	1.0	3.8	1.0	2.5	1.0	2.2
BDNF	1.0	6.3	0.6	3.3	2.0	4.4
GDNF	1.0	5.7	1.3	2.8	0.9	1.0
NGF	1.0	5.3	0.6	2.0	0.9	1.2

4. Analysis of Angiogenesis-Inducing Ability, Cell Proliferative Ability, and Cell Migration Ability

[0244] The angiogenesis-inducing ability, cell proliferative ability, cell migration ability of the 5^{th}-generation dental pulp, bone marrow, and adipose membrane-separated cells were examined in vitro. With regard to angiogenesis-inducing ability, the aforementioned different types of cells dispersed on an EGM-2 (Lonza) medium were each subjected to a three-dimensional culture on matrigel, and the cultured cells were compared regarding lumen formation ability. With regard to cell proliferative ability, using TetraColor One (Seikagaku Biobusiness Corporation), cell proliferative ability was measured by stimulation with 10% FBS or 100 ng/ml G-CSF. Moreover, cell migration ability to G-CSF was analyzed by real-time horizontal chemotaxis analysis using TAXIScan-FL. Specifically, a channel optimized to the size of cells (8 μm) was formed between silicone having pores with a pore size of 6 μm and a glass plate. Thereafter, membrane-separated pig dental pulp, bone marrow and adipose cells, and unseparated pig dental pulp, bone marrow and adipose test cells, were each poured in one side of the channel (1 μl each; cell density: 10^5 cells/ml). Various types of migration factors (10 ng/μl) were each poured into the opposite side thereof to form a certain concentration gradient. Based on video images of migration, the number of migrating cells was counted every 30 minutes until 24 hours after initiation of the operation.

[0245] With regard to angiogenesis induction, all of the membrane-separated dental pulp, bone marrow, and adipose-derived cells were observed to form a cord-like structure on matrigel 5 hours after initiation of the operation, thereby exhibiting ability to differentiate into vascular endothelial cells. On the other hand, the unseparated test cells did not form such a cord-like structure even after observation for a long period of time.

[0246] A graph regarding a comparison of cell proliferative ability in which fetal bovine serum was used is shown in FIG. **19**, and a graph regarding a comparison of cell proliferative ability in which G-CSF was used is shown in FIG. **20**. The cell proliferative ability of the membrane-separated cells examined using FBS and G-CSF was higher than that of the unseparated test cells. A graph regarding the measurement of

the number of migrating cells using G-CSF is shown in FIG. **21**. It was found that the cell migration ability of the membrane-separated cells examined using G-CSF was highest among all types of unseparated test cells.

Example 7

Method for Directly Separating Stem Cells from Fresh Dental Pulp and Adipose Tissues by Membrane

[0247] Whether stem cells can be directly separated from dental pulp and adipose tissues by a membrane separation method without performing enzyme digestion, as with other cells, was examined. As a membrane separation device, Cellculture Insert (a surface-modified polycarbonate base material membrane Transwell (registered trademark) Inserts; 2×10^5 pores/cm^2, pore size: 8 μm, diameter of bottom surface: 6.4 mm, diameter of opening portion: 11.0 mm, height: 17.5 mm) (Corning), used an upper structure, was inserted into a 24-well plate (diameter: 15.0 mm, diameter of opening portion: 15.0 mm, height: 22.0 mm) (Falcon), used as a lower structure, and the thus prepared device was used as a membrane separation device. As in the case of Example 6 as described above, this polycarbonate base material membrane had also been subjected to a treatment for imparting a non-cell-adhesive property thereto, and a separation membrane was then prepared. The prepared separation membrane was incorporated into the membrane separation device. Thereafter, minced fresh dog dental pulp tissues or adipose tissues were left at rest on the upper portion of this polycarbonate membrane, meanwhile, G-CSF (final concentration: 100 ng/ml) was added into Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS in 24 wells of the lower structure of the membrane. Twenty-four hours later, the G-CSF was removed, and the medium was replaced with another DMEM containing 10% FBS, followed by performing a culture. After the cells had become 70% confluent, they were subcultured.

[0248] The states of dental pulp stem cells and adipose stem cells that have migrated and adhered 24 hours after initiation of the operation are shown in FIG. **23**. As a result, it was found that the cells could also be separated also from dental pulp tissues and adipose tissues under the same conditions as those for separation from the test cells and were collected in the lower portion of the membrane.

Example 8

1. Measurement Methods

(1) Electron Spectroscopy for Chemical Analysis (ESCA) Measurement

[0249] Three points on each of the inner surface and outer surface of a separation membrane were measured. The measurement sample was rinsed with ultrapure water, was then dried at a room temperature at 0.5 Ton for 10 hours, and was then subjected to measurement. The measurement device and measurement conditions are the following:

[0250] Measurement device: ESCLAB220iXL

[0251] Excitation X-ray: monochromatic AlKa 1,2 ray (1486.6 eV)

[0252] X-ray diameter: 0.15 mm

[0253] Photoelectron escape angle: 90° (inclination of detector to sample surface).

[0254] Moreover, by analyzing the separation membrane by an elementary analysis method, the amount of a hydrophilic polymer on the surface of the separation membrane can be calculated, for example, based on the amount of nitrogen (a (atom number %)) and the amount of sulfur (b (atom number %)) and the like. In the case of polyacrylonitrile, a calibration curve of film was prepared based on the ratio of the peak strength of C≡N derived from nitrile groups around 2,200 cm^{-1} (ACN) and ACO in the same manner as described above, and the ratio of an internal vinyl acetate unit amount was then obtained.

(2) Method of Measuring Hydrophilic Polymer Distribution According to Infrared Absorption Spectrometry

[0255] The separation membrane was rinsed with ultrapure water, and was then dried at a room temperature at 0.5 Ton for 10 hours. The surface of the thus dried separation membrane was measured by microscopic ATR method using IRT-3000 manufactured by JASCO. A visual field region (aperture) was set at 100 μm×100 μm, the cumulated number per single point was set at 30, and the aperture was moved by each 3 μm so that the measurement was carried out at a total of 25 points consisting of 5 points in the longitudinal direction and 5 points in the vertical direction. Moreover, based on the measurement of a difference spectrum from a surface-not-modified membrane, the amount of the adhered hydrophilic polymer was calculated.

(3) Method of Testing Adhesion of Human Platelets to Membrane

[0256] A separation membrane was attached to a Falcon (registered trademark) tube (18 mmϕ, No. 2051) cut into a cylindrical shape such that the surface of the membrane to be evaluated could be inside the cylinder. Then, the space was filled with paraffin. The inside of this cylindrical tube was washed with a normal saline, and was then filled with a normal saline. Venous blood was collected from a healthy volunteer, and heparin was immediately added to the collected blood to a concentration of 50 U/ml. The normal saline was discarded from the cylindrical tube. Thereafter, 1.0 ml of the blood was added into the cylindrical tube within 10 minutes after blood collection, and it was then shaken at 37° C. for 1 hour. Subsequently, a hollow fiber membrane was washed with 10 ml of a normal saline, blood component was then immobilized thereon with a 2.5 weight % glutaraldehyde normal saline, and the membrane was then washed with 20 ml of distilled water. The washed separation membrane was dried under a reduced pressure at an ordinary temperature at 0.5 Torr for 10 hours. Thereafter, using a double stick tape, the resulting separation membrane was attached on a sample stand of a scanning electron microscope. After that, a thin membrane of Pt—Pd was formed on the surface of the hollow fiber membrane by sputtering to prepare a sample. The inner surface of this separation membrane was observed at a magnification of 1,500 times under field emission scanning electron microscope (S800, manufactured by Hitachi), and the number of platelets adhered to a single visual field (4.3×10^3 μm^2) was counted. A mean value of platelets adhered to 10 different visual fields around the center in the longitudinal direction of the hollow fiber was defined as the number of adhering platelets (platelets/4.3×10^3 μm^2).

[0257] The number of platelets adhered to a material having a good platelet adhesion-suppressing property is 40 or

less (platelets/4.3×10^3 μm^2), preferably 20 or less (platelets/4.3×10^3 μm^2), and more preferably 10 or less 0 (platelets/4.3×10^3 μm^2) or less.

(4) Evaluation of Cell Permeation

[0258] The separation membrane attached to Cellculture Insert manufactured by BD was used, and "Mesenchymal Stem Cell" manufactured by PromoCell was used herein as mesenchymal stem cells. Mesenchymal Stem Cell Adipogenic Differentiation Medium #C-28011, Ready-to-use (a medium used for proliferation of mesenchymal stem cells) was used as a medium for differentiation and culture, and this medium was placed in the lower portion of the above-modified Cellculture Insert manufactured by BD. On the other hand, the above-mentioned cells and a medium for culture (Mesenchymal Stem Cell Expansion Media, Human/Mouse, StemXVivo) were placed in the upper portion of the modified Cellculture Insert. Moreover, G-CSF was further added into the medium in the lower portion to a final concentration of 100 ng/ml, and it was then left at 37° C. in a CO_2 incubator for 12 hours. The number of cells dropped from the upper portion to the lower petri dish for 12 hours was counted under a phase contrast microscope.

[0259] The water absorption percentage of each of a PET membrane, a polycarbonate membrane, a PP membrane and a polysulfone membrane was 2% or less, although the water absorption percentage of a polyamide membrane was 4.2%. The used base material membrane was immersed in water at 23° C. for 24 hours, and an increased weight was defined as the water absorption percentage of a polymer.

[0260] In accordance with such water absorption percentage, using a membrane consisting of polycarbonate and a polyamide membrane ("Nylon 66"), a surface treatment was carried out under conditions as described in the table below. A membrane portion of the "Cellculture Insert" was appropriately replaced with the used membrane, and cells were then allowed to migrate using a G-CSF migration factor. Thereafter, the number of cells that permeated through the membrane and adhered to the lower petri dish was then counted. We found that, in the case of using a membrane consisting of PET, the adhesive property of the cells was suppressed in an ethanol (EtOH) addition system, and that a large number of cells dropped and adhered to the lower petri dish. Also, it was found that, in the case of using a polyamide membrane, a cell adhesion-suppressing property was expressed in an EtOH-non-addition system.

[0261] The amount of a hydrophilic polymer bound to the surface was appropriately analyzed and was obtained by an elementary analysis method, ESCA, and an IR method. The following hydrophilic polymers were used:

[0262] PVP: polyvinyl pyrrolidone (K90, K30), Tokyo Chemical Industry Co., Ltd.

[0263] PVA: polyvinyl alcohol (manufactured by Kuraray Co., Ltd.)

[0264] VA64: polyvinyl pyrrolidone/polyvinyl acetate copolymer (Kollidon VA64, manufactured by BASF).

Results of Treatment of Polycarbonate Membrane

[0265] Cellculture Insert comprising a polycarbonate membrane having pores with a pore diameter of 8 μm was used, and the surface of the membrane was modified by immersing it in aqueous solutions having different conditions, sealing it, and then irradiating it with a γ-ray (25 kGy). Thereafter, human mesenchymal stem cells were dispersed on the upper portion of the membrane (1×10^4 cells/100 μl), and the number of cells adhered to the lower portions of 24 wells was then measured under phase contrast microscope, thereby evaluating separation performance. The results are shown in the following Table 11.

TABLE 11

surface modification	the amount of a hydrophilic polymer adhered (wt %)	the number of cells that permeated through the membrane
untreated	0	2
VA64 1000 ppm + EtOH 0.1 wt %	31	450
VA64 1000 ppm	1.5	210
VA64 10 ppm + EtOH 0.1 wt %	1.6	190
VA64 1000 ppm + EtOH 0.2 wt %	35	500
VA64 1000 ppm + EtOH 0.01 wt %	11	330
PVP(K90)1000 ppm + EtOH 0.1 wt %	16	350
PVP(K90) 100 ppm + EtOH 0.01 wt %	1.1	35

Results of Treatment of Polyamide Membrane

[0266] The membrane portion of the Cellculture Insert was replaced with a polyamide membrane having pores with a pore diameter of 8 μm, and the surface of the replaced membrane was modified by immersing it in aqueous solutions having different conditions, sealing it, and then irradiating it with a γ-ray (25 kGy). Thereafter, human mesenchymal stem cells were dispersed on the upper portion of the membrane (1×10^4 cells/100 μl), and the number of cells adhered to the lower portions of 24 wells was then measured under phase contrast microscope, thereby evaluating separation performance. The results are shown in the following Table 12.

TABLE 12

surface modification	amount of a hydrophilic polymer adhered (wt %)	the number of cells that permeated through the membrane
untreated	0	40
VA64 1000 ppm + EtOH 0.1 wt %	16	320
VA64 1000 ppm	33	480
VA64 10 ppm	2.8	192
VA64 100 ppm	15	346
VA64 100 ppm + EtOH 0.1 wt %	11	311
PVP(K90) 1000 ppm	18	352
PVP(K90) 100 ppm + EtOH 0.01 wt %	1.3	56

Influence of Hydrophilic Polymer

[0267] Cellculture Insert comprising a polycarbonate membrane having pores with a pore diameter of 8 μm was used, and the surface of the membrane was modified by immersing it in aqueous solutions having different conditions, sealing it, and then irradiating it with a γ-ray (25 kGy). Thereafter, human mesenchymal stem cells were dispersed on the upper portion of the membrane (1×10^4 cells/100 μl), and the number of cells adhered to the lower portions of 24

wells was then measured under phase contrast microscope, thereby evaluating separation performance. The results are shown in the following Table 13.

TABLE 13

surface modification	amount of a hydrophilic polymer adhered (wt %)	the number of cells that permeated through the membrane
Untreated	0	13
PVP K90 1000 ppm + EtOH 0.1 wt %	18	311
PVP K90 1000 ppm	2.2	149
PVP K30 1000 ppm + EtOH 0.1 wt %	13	255
PVP K30 1% + EtOH 0.1 wt %	16	321
PVA417 1000 ppm + EtOH 0.1 wt %	17	330
PVA417 1000 ppm	1.8	116
PEG20,000 1000 ppm + EtOH 0.1 wt %	4	123
VA64 1000 ppm + EtOH 0.1 wt %	33	436
VA64 1000 ppm	1.9	132
PVP(K90) 100 ppm + EtOH 0.01 wt %	1.3	43

Confirmation of Platelet Adhesive Property

[0268] Using a film consisting of PET and having no pores, the number of platelets adhered thereto was counted under various conditions. The results are shown in the following Table 14.

TABLE 14

surface modification	the amount of a hydrophilic polymer adhered (wt %)	the number of platelets adhered/ 4.3×10^3 μm^2
Untreated	0	230
PVP K90 1000 ppm + EtOH 0.1 wt %	20	9
PVP K90 1000 ppm	3.1	37
PVP K30 1000 ppm + EtOH 0.1 wt %	15	22
PVP K30 1% + EtOH 0.1 wt %	16	19
PVA417 1000 ppm + EtOH 0.1 wt %	15	18
PVA417 1000 ppm	2.2	33
PEG20,000 1000 ppm + EtOH 0.1 wt %	4	19
VA64 1000 ppm + EtOH 0.1 wt %	29	3
VA64 1000 ppm	22	31
PVP(K90) 100 ppm + EtOH 0.01 wt %	1.2	157

Results of Treatment of Polycarbonate Membrane

[0269] Cellculture Insert comprising a polycarbonate membrane having pores with a pore diameter of 5 μm was used, and the surface of the membrane was modified by immersing it in aqueous solutions having different conditions, sealing it, and then irradiating it with a γ-ray (25 kGy). Thereafter, human dental pulp cells at the 2nd passage of culture were dispersed on the upper portion of the membrane (2×10^4 cells/100 μl), and G-CSF (final concentration: 100 ng/ml) was added into Dulbecco's modified Eagle's medium (DMEM) containing 10% human serum in the 24 wells of the lower structure thereof. Forty-eight hours later, the G-CSF was removed, and the medium was replaced with another DMEM containing 10% human serum. Thereafter, the number of cells adhered to the lower portion of the 24 wells was counted under phase contrast microscope. The results are shown in Table 15.

TABLE 15

surface modification	amount of a hydrophilic polymer adhered (wt %)	cell permeation rate (%)
untreated	0	0.2
VA64 1000 ppm + EtOH 0.1 wt %	28	4.5
VA64 1000 ppm	1.7	2.1
VA64 10 ppm + EtOH 0.1 wt %	1.9	1.9
VA64 1000 ppm + EtOH 0.2 wt %	31	4.4
VA64 1000 ppm + EtOH 0.01 wt %	14	3.3
PVP(K90)1000 ppm + EtOH 0.1 wt %	17	6.5
PVP(K90) 100 ppm + EtOH 0.01 wt %	1.3	0.3

Results of Treatment of Polyamide Membrane

[0270] Subsequently, the membrane portion of the Cellculture Insert was replaced with a polyamide membrane having pores with a pore diameter of 5 μm, and the surface of the replaced membrane was modified by immersing it in aqueous solutions having different conditions, sealing it, and then irradiating it with a γ-ray (25 kGy). Thereafter, the same operations as those described above were carried out. The obtained results are shown in Table 16.

TABLE 16

surface modification	amount of a hydrophilic polymer adhered (wt %)	cell permeation rate (%)
untreated	0	0.4
VA64 1000 ppm + EtOH 0.1 wt %	15	3.2
VA64 1000 ppm	31	4.8
VA64 10 ppm	2.2	1.9
VA64 100 ppm	16	3.5
VA64 100 ppm + EtOH 0.1 wt %	12	3.1
PVP(K90) 1000 ppm	21	3.5
PVP(K90) 100 ppm + EtOH 0.01 wt %	1.2	0.5

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<223> OTHER INFORMATION: Porcine forward primer for beta-actin

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<210> SEQ ID NO 76
<211> LENGTH: 20
<212> TYPE: DNA
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<223> OTHER INFORMATION: Porcine reverse primer for beta-actin

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acgtcgcact tcatgatcga 20

1. A membrane separation culture device comprising:

an upper structure comprising a vessel in which at least a portion of the bottom thereof is formed with a separation membrane having pores that allow stem cells to permeate therethrough; and

a lower structure comprising a vessel that retains a fluid in which the membrane of the upper structure is immersed.

2. The membrane separation culture device according to claim **1**, wherein the separation membrane comprises:

a base material membrane consisting of a hydrophobic polymer; and

a functional layer formed by allowing one or more hydrophilic polymers selected from a vinyl pyrrolidone polymer, a polyethylene glycol polymer and a vinyl alcohol polymer to bind to the surface of the base material membrane via a covalent bond; wherein

weight percentage of the hydrophilic polymer(s) constituting the functional layer is 1.5% to 35% based on the total weight of the separation membrane.

3. The membrane separation culture device according to claim **1**, wherein the size of the pore is 3 μm to 10 μm and the density of the pore is 1×10^5 to 4×10^6 pores/cm^2.

4. The membrane separation culture device according to claim **1**, comprising a plurality of the upper structures, and

further comprising a frame body accommodated in the lower structure and comprises a plate-like member having a plurality of holes each established to lock the plurality of the upper structures.

5. The membrane separation culture device according to claim **1**,

comprising a plurality of the upper structures, and further comprising a frame body accommodated in the lower structure and comprises a plate-like member having a plurality of holes each established to lock the plurality of the upper structures, wherein

the lower structure comprises a plurality of vessels each corresponding to the plurality of the upper structures.

6. The membrane separation culture device according to claim **4**, wherein the plurality of the upper structures have membranes each having a different pore size and/or a different pore density.

7. The membrane separation culture device according to claim **1**, further comprising a lid structure that covers or hermetically seals the upper structure and the lower structure.

8. The membrane separation culture device according to claim **7**, wherein the lid structure further comprises a gas exchanger comprising a gas inlet port and a gas discharge port.

9. The membrane separation culture device according to claim **7**, wherein at least a portion of the lid structure is formed with a membrane having pores whose pore size is 1 to 100 nm.

10. The membrane separation culture device according claim **7**, wherein a hermetic sealing elastic body is established between the lid structure and the lower structure.

11. The membrane separation culture device according to claim **7**, further comprising a temperature control system containing a temperature-measuring device and a temperature-controlling device.

12. The membrane separation culture device according to claim **1**, wherein the lower structure further comprises a medium replacement system comprising a medium inlet port and a medium outlet port.

13. A membrane separation culture kit comprising the membrane separation culture device according to claim **1** and cell migration factor(s) to be poured into the lower structure.

14. The kit according to claim **13**, wherein the cell migration factor(s) are one or more selected from SDF-1, G-CSF, bFGF, TGF-β, NGF, PDGF, BDNF, GDNF, EGF, VEGF, SCF, MMP3, Slit, GM-CSF, LIF, HGF, S1P, protocatechuic acid, and serum.

15. The kit according to claim **13**, wherein the concentration of the cell migration factor(s) is 1 ng/ml to 500 ng/ml.

16. The kit according to claim **13**, further comprising serum to be poured into the lower structure and wherein the cell migration factor is G-CSF or bFGF.

17. A method of separating stem cells with the membrane separation culture device according to claim **1**, comprising:

dispersing test cells or test tissues on the membrane of the upper structure;

filling the vessel as a lower structure with a medium containing cell migration factor(s); and

causing the membrane of the upper structure to contact the medium in the lower structure.

18. The method according to claim **17**, wherein the cell migration factor(s) are one or more selected from SDF-1, G-CSF, bFGF, TGF-13, NGF, PDGF, BDNF, GDNF, EGF, VEGF, SCF, MMP3, Slit, GM-CSF, LIF, HGF, SIP, protocatechuic acid, and serum.

19. The method according to claim **17**, wherein concentration of the cell migration factor(s) is 1 ng/ml to 500 ng/ml.

20. The method according to claim **17**, wherein the test cells are dispersed at a density of 3×10^2 cells to 3×10^4 cells per mm^2 of the separation membrane.

21. The method according to claim **17**, wherein the stem cells are dental pulp stem cells, the cell migration factor is G-CSF or bFGF, the concentration of the G-CSF or bFGF is 50 to 150 ng/ml, the test cells are dispersed at a density of 3×10^2 to 1.5×10^3 cells per mm^2 of the separation membrane, or the test tissues are left at rest at a density of 0.1 mg to 1 mg per mm^2 of the separation membrane, and serum is added to a medium containing the cell migration factor at a volume percentage of 5% to 20% based on the volume of the medium.

22. The method according to claim **17**, wherein the stem cells are bone marrow stem cells or adipose stem cells, the cell migration factor is G-CSF or bFGF, the concentration of the G-CSF or bFGF is 50 to 150 ng/ml, the test cells are dispersed at a density of 3×10^2 to 1.5×10^3 cells per mm^2 of the separation membrane, or the test tissues are left at rest at a density of 0.1 mg to 1 mg per mm^2 of the separation membrane, and serum is added to a medium containing the cell migration factor at a volume percentage of 5% to 20% based on the volume of the medium.

23. A separation membrane comprising:

a base material membrane consisting of a hydrophobic polymer; and

a functional layer formed by allowing one or more hydrophilic polymers selected from a vinyl pyrrolidone polymer, a polyethylene glycol polymer and a vinyl alcohol polymer to bind to the surface of the base material membrane via a covalent bond; wherein

weight percentage of the hydrophilic polymer(s) constituting the functional layer is 1.5% to 35% based on the total weight of the separation membrane.

24. The separation membrane according to claim **23**, wherein the base material membrane has pores with a pore size of 1 to 10 μm and the base material membrane is used for cell separation.

25. The separation membrane according to claim **23**, wherein the hydrophobic polymer is selected from the group consisting of a sulfone polymer, an amide polymer, a carbonate polymer, an ester polymer, a urethane polymer, an olefin polymer, and an imide polymer.

26. The separation membrane according to claim **23**, which separates cells by permeation.

27. A method of producing the separation membrane according to claim **23**, comprising:

immersing a base material membrane consisting of a hydrophobic polymer having a water absorption percentage of 2% or less in a treating aqueous solution containing one or more hydrophilic polymers selected from a vinyl pyrrolidone polymer, a polyethylene glycol polymer and a vinyl alcohol polymer at a concentration of 10 to 2000 ppm, and also containing a 0.01% to 0.2% alcohol; and

irradiating the base material membrane with a high-energy beam to modify the surface of the membrane to have a protein adhesion-suppressing property and a cell adhesion-suppressing property.

28. A method of producing the separation membrane according to claim **23**, comprising:

immersing a base material membrane consisting of a hydrophobic polymer having a water absorption percentage of more than 2% in an aqueous solution containing one or more hydrophilic polymers selected from

a vinyl pyrrolidone polymer, a polyethylene glycol polymer and a vinyl alcohol polymer at a concentration of 10 to 2000 ppm; and

irradiating the base material membrane with a high-energy beam to modify the surface of the membrane to have a protein adhesion-suppressing property and a cell adhesion-suppressing property.

29. The method according to claim **27**, wherein the hydrophobic polymer is selected from the group consisting of a sulfone polymer, an amide polymer, a carbonate polymer, an ester polymer, a urethane polymer, an olefin polymer, and an imide polymer.

30. A method of modifying a surface of a molded body, comprising:

immersing a molded body having a water absorption percentage of 2% or less in a treating aqueous solution containing one or more hydrophilic polymers selected from a vinyl pyrrolidone polymer, a polyethylene glycol polymer and a vinyl alcohol polymer at a concentration of 10 to 2000 ppm, and also containing a 0.01% to 0.2% alcohol; and

irradiating the molded body with a high-energy beam to modify the surface of the molded body to have a protein adhesion-suppressing property and a cell adhesion-suppressing property.

31. A method of modifying a surface of a molded body comprising:

of immersing a molded body having a water absorption percentage of more than 2% in an aqueous solution containing one or more hydrophilic polymers selected from a vinyl pyrrolidone polymer, a polyethylene glycol polymer and a vinyl alcohol polymer at a concentration of 10 to 2000 ppm; and

irradiating the molded body with a high-energy beam to modify the surface of the molded body to have a protein adhesion-suppressing property and a cell adhesion-suppressing property.

32. A method of producing the separation membrane according to claim **23** by a method of modifying a surface of a molded body comprising:

immersing a molded body having a water absorption percentage of 2% or less in a treating aqueous solution containing one or more hydrophilic polymers selected from a vinyl pyrrolidone polymer, a polyethylene glycol polymer and a vinyl alcohol polymer at a concentration of 10 to 2000 ppm, and also containing a 0.01% to 0.2% alcohol; and

irradiating the molded body with a high-energy beam to modify the surface of the molded body to have a protein adhesion-suppressing property and a cell adhesion-suppressing property.

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